STEROIDOGENIC ACUTE REGULATORY (StAR) PROTEIN IN BOVINE ADRENAL STERIODOGENESIS

HUI WANG

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I dedicate this thesis to my mother and father and
to the memory of my grandmother (1918-2001).

DECLARATION OF ORIGINALITY

I declare that the composition of this thesis and the work presented herein is my own. Work performed by others as a part of collaboration are indicated in the text.

Hui Wang

CONTENTS

ABSTRACT

Both acute and chronic steroidogenesis are regulated by adrenocorticotropic hormone (ACTH), a principal regulator of the adrenal cortex. A number of studies have demonstrated that Steroidogenic Acute Regulatory (StAR) protein plays a crucial role in facilitating cholesterol transfer from the outer to the inner mitochondrial membrane, where the first step of cholesterol conversion to steroid hormones occurs. This work has evaluated the relationships between the expression of StAR protein and signalling pathways of ACTH-induced steroidogenesis in primary cultures of bovine adrenal zona fasciculata (ZF) cells.

A novel sheep anti-bovine peptide StAR polyclonal antibody has been characterised and optimised for Western immunoblotting. A newly formulated protocol based on enhanced chemiluminescence methodology provided a linear, reproducible and sensitive approach to detect and quantify StAR protein by molecular image analysis.

The expression level of StAR protein after ACTH treatment of adrenal ZF cells showed that levels of StAR were insensitive to ACTH in freshly isolated cells due to the high initial levels of the protein. The cell responsiveness to ACTH was remarkable, however, after the basal levels of StAR protein diminished to a relatively lower levels after 2 days of culture. Concentration-response curves demonstrated that, in general, increasing concentrations of ACTH resulted in increasing in cortisol output in parallel with increases of cyclic adenosine 3', 5'-monophosphate (cAMP) production; the maximal effects of ACTH on cortisol and cAMP levels exhibited with 10^{-8} M ACTH treatment after both 1 and 6 hr. Marked changes in StAR protein occurred at 6 hr, attaining a maximal level with 10^{-8} M ACTH. Interestingly, at 1 and 6 hr the elevation of cortisol levels were significantly altered at 10^{-12} M ACTH without any notable increase in cAMP levels. The time courses for ACTH treatment showed that at 10^{-8} M ACTH (a supraphysiological concentration) there was a strong positive correlation between cortisol and StAR protein induction, suggesting that newly synthesised StAR protein may be a major mediator for steroidogenesis. On the other hand, at 10^{-12} M ACTH (a physiological concentration) cortisol secretion rose apparently prior to the measurable changes in total StAR protein, and the cortisol changes were poorly correlated with StAR protein. All these profiles clearly suggested that steroidogenesis may at least

partially rely on pre-existing StAR protein and a cAMP-independent signalling pathway may also participate in steroidogenesis, particularly for physiologically relevant doses of ACTH.

The roles of phospholipase A_2 (PLA_2) and arachidonic acid (AA) signalling pathways in ACTH-stimulated cortisol secretion were evaluated using various selective inhibitors, including 4-bromophenacyl bromide for PLA_2, nordihydroguaiaretic acid for lipoxygenase and indomethacin for cyclooxygenase. The results suggested that cortisol induction by 10^{-8} M ACTH is also mediated by PLA_2-AA and/or AA metabolites in addition to the cAMP pathway. More importantly, the PLA_2-AA signalling system seemed potentially a key second messenger system in response to 10^{-12} M ACTH. Meanwhile, the total StAR protein levels exhibited no significant alteration in the presence of the various inhibitors.

Further investigation showed that phorbol-12-myristate 13-acetate (PMA), a protein kinase C activator, significantly increased the expression of StAR protein ($P<0.05$). However, it poorly promoted cortisol output, possibly due to a lack of appropriate Ca^{2+} mobilisation. Chronically (24 hr), PMA increased StAR protein levels ($P<0.01$) but attenuated cortisol production in the presence of ACTH (10^{-8} M), implying that an inhibitory effect of PMA on other components of steroidogenic pathways was likely. Furthermore, bisindolylmaleimide I, a highly selective protein kinase C inhibitor, resulted in significant inhibitory effect on cortisol levels induced by 10^{-12} M and 10^{-8} M ACTH, especially at 1 hr, and appeared not to affect the level of StAR protein.

In summary, very acute increases in cortisol may well be independent of the changes in total StAR protein levels. The *de novo* synthesis of StAR protein provides, however, a continuous source of the labile protein that promotes further elevated levels of cortisol output. cAMP and AA cascades are dual signalling pathways mediating ACTH-stimulated cortisol output. Overall, it is suggestive that there are at least two fundamental mechanisms for steroidogenesis, i.e. the activation of second messenger networks and the regulation of the protein(s) for initiating steroidogenesis via a rapid non-genomic response (post-translational modification).

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Abbreviations

AA	arachidonic acid
AC	adenylate cyclase
ACTH	adenocorticotrophic hormone
ACTH-R	adenocorticotropic hormone receptor
AmpB	amphotericin
AMPS	ammonium persulfate
Ang II	angiotensin II
ANOVA	analysis of variance
ARTISt	arachidonic acid-related thioesterase involved in steroidogenesis
4-BPB	4-bromophenacyl bromide
BSA	bovine serum albumin
°C	Celsius degree
Ca^{2+}	calcium ion
$[Ca^{2+}]_c$	cytoplasmic free calcium concentration
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic adenosine 3', 5'-monophosphate
CE	cholesterol ester
CEH	cholesterol ester hydrolase
CHX	cycloheximide
COX	cyclooxygenase
CPSR-1	controlled process serum repalcement-1
CRH	corticotropin-releasing hormone
CS	contact site
CYP11A	cytochrome P450 side-chain cleavage enzyme
CYP11B	cytochrome P450 11β-hydroxylase (in bovine ZFR) or cytochrome P450 aldosterone synthase (in bovine ZG)
CYP11B1	cytochrome P450 11β-hydroxylase (in human and rat ZFR)
CYP11B2	cytochrome P450 aldosterone synthase (in human and ratZG)
CYP17	cytochrome P450 17α-hydroxylase
CYP19	cytochrome P450 aromatase
CYP21	cytochrome P450 21-hydroxylase

CV	coefficient of variation
2-D	two-dimensional
DAG	1, 2-diacylglycerol
DAX-1	dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1
DBI	diazepam binding inhibitor
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EBSS	Earle's balanced salt solution
EC_{50}	half effective concentration
ECL	enhanced chemiluminescence
ED_{50}	median effective dose
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
FAD	flavin-adenine dinucleotide
FAF	fatty acid free
FSH	follicle-stimulating hormone
g	gravity
GF109203X	bisindolylmaleimide I
GTPase	any enzyme that hydrolyses GTP to GDP and ortophosphate
hCG	human chorionic gonadotropin
HCl	hydrochloric acid
HDL	high density lipoprotein
HETE	hydroxyeicosatetraenoic acid
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme
H_20_2	hydrogen peroxide
HPETE	hydroperoxyeicosatetraenoic acid
hr	hour
HRP	horseradish peroxidase
3β-HSD	3β-hydroxysteroid dehydrogenease
17β-HSD	17β-hydroxysteroid dehydrogenase.
IC_{50}	the median inhibitory concentration of an agent
I_{AC}	K^+ current
Ig	immunoglobulin
IM	inner membrane of mitochondria

INDO	indomethacin
IP_3	inositol (1,4,5) triphosphate
i.v.	intravenous
kDa	kilodalton
kb	kilobase
LDL	low density lipoprotein
LOX	lipoxygenase
LT	leukotriene
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
NDGA	nordihydroguaiaretic acid
NADH	nicotinamide-adenine dinucleotide (reduced)
NADPH	nicotinamide-adenine dinucleotide phosphate(reduced)
nm	nanometre
nM	nanomolar
OM	outer membrane of mitochondria
P value	the probability of the statistic significance
PAGE	polyacrylamide gel electrophoresis
PBR	peripheral benzodiazepine receptor
PBS	phosphate-buffered saline
PG	prostaglandin
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
$cPLA_2$	cytosolic phospholipase A_2
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate 13-acetate
P/S	pehicillin/streptomycin
PVDF	polyvinylidene difluoride

r	correlation coefficient
RIA	radioimmunoassay
RNA	rideoxyribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SAP	steroidogenesis activator polypeptide
SCP_2	sterol carrier protein 2
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SF-1	steroidogenic factor-1
TEMED	N,N, N',N'-tetramethylethylenediamine
TPA	12-O-tetrasecanoylphorbol-13-acetate
µg	microgram
µl	microlitre
v/w	volume verse weight
ZG	zona glomerulosa
ZF	zona fasciculata
ZFR	zona fasciculata-reticularis
ZR	zona reticularis

CHAPTER 1 INTRODUCTION

1.1 THE ADRENAL GLAND

1.1 .1 Functional anatomy of the adrenal gland

The main steroidogenic glands of mammals are the adrenal gland and gonads (testis and ovary). Fig. 1-1-1 illustrates that the adrenal gland consists of two embryologically and functionally distinct tissues: the cortex and the medulla. Conventionally, the adrenal cortex is further divided into three concentric zones, varying in both their morphological features and the steroid hormones which they secrete. The zona glomerulosa (ZG) cells produce the mineralocorticoid aldosterone, zonae fasciculata (ZF) and reticularis (ZR) secrete the glucocorticoids (cortisol and corticosterone) and 19-carbon steroids (Orth et al., 1992). Recent studies have shown a novel layer between ZG and ZF in the rat (Mitani et al., 1994), which was proposed as a undifferentiated zone containing stem cells (Mitani et al., 1999). The adrenal medulla is the central part of the adrenal gland, comprising almost entirely chromaffin cells. They secrete catecholamines including adrenaline (epinephrine), noradrenaline (norepinephrine), and small amounts of dopamine (Landsberg and Young, 1992).

1.1.2 Control of the adrenal cortex

Fig. 1-1-2 shows that the secretion of glucocorticoids is principally regulated by the hypothalamus-pituitary-adrenal (HPA) axis and its negative feedback loop. In response to stress the hypothalamus secretes corticotrophin-releasing hormone (CRH) which stimulates the release of adenocorticotrophic hormone (ACTH) from the adrenohypophysis (anterior pituitary). Subsequently, ACTH circulates to the adrenal cortex, stimulating glucocorticoids secretion (Orth et al., 1992). The major circulating mineralocorticoid, aldosterone, is produced by the adrenal ZG which is controlled by multiple factors that have complex regulatory interactions (Quinn and Williams, 1988), of which the renin-angiotensin system and potassium ions are of key importance (Orth et al., 1992).

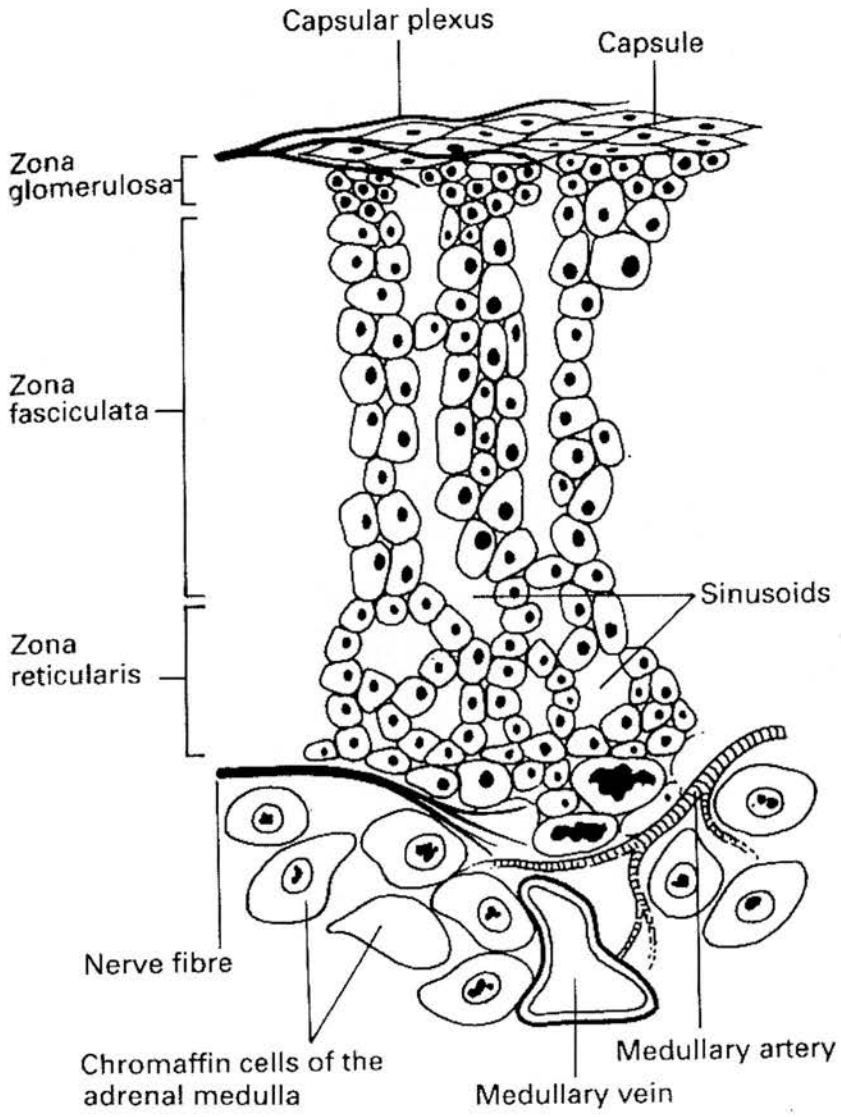

Fig. 1-1-1 Schematic structure of the adrenal cortex and medulla (Jeffcoate, 1993).

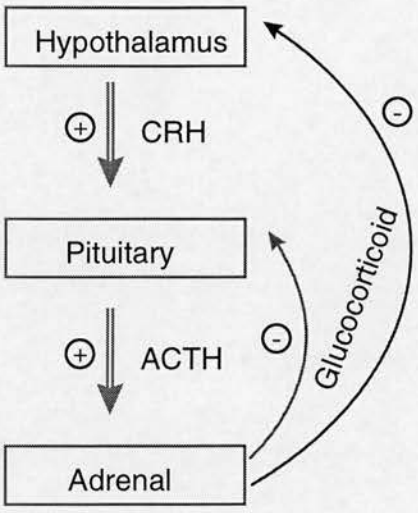

Fig. 1-1-2 Hormonal regulation of adrenal glucocorticoid via HPA axis and the feedback of loop. ⊕: Positive control; ⊖: Negative control.

1.2 BIOSYNTHESIS OF STEROID HORMONES

1.2.1 Cholesterol translocation

Unlike polypeptide hormone-secreting cells such as pituitary cells, steroidogenic cells do not tend to store steroid hormones. This is because lipophilic steroids can diffuse more or less freely across organellar and cellular membranes. Thus, instead of accumulating the end products, steroidogenic cells accumulate large amounts of steroid precursor, cholesterol. These cells are able to use one or more of three sources of cholesterol, i.e. intracellular stores, circulating lipoproteins (LDL and HDL) and *de novo* synthesis (Cherradi et al., 1998a; Jefcoate et al., 1992; Kovanen et al., 1979).

In mammals steroids are synthesised from a common substrate, cholesterol. The initial phase of steroid synthesis is the mobilisation of cholesterol from cellular compartments such as lipid droplets to the outer mitochondrial membrane (i. e. transfer step 1), which may be via a process involving cytoskeletal components (e.g.

microfilaments and microtubules), calmodulin-Ca^{2+}, and perhaps also a sterol-binding protein (Crivello and Jefcoate, 1980; Simpson and Waterman, 1983).

Subsequently, cholesterol is transferred from the outer to the inner mitochondrial membrane (i. e. transfer step 2) where the cytochrome P450 side chain cleavage enzyme (CYP11A) resides (Cherradi et al., 1998a; Jefcoate et al., 1992). CYP11A has been observed at the matrix side of the inner mitochondrial membrane by immunochemical staining together with electron microscopic analysis (Mitani et al., 1982). On the other hand, the synthesis of the first product of steroidogenesis, pregnenolone (Stone and Hechter, 1954), is independent of prior hormonal stimulation when adrenal mitochondria are incubated with 25-hydroxycholesterol which readily passes through mitochondria membranes (Jefcoate et al., 1974; Simpson and Waterman, 1983). This result is indicative that CYP11A activity *per se* is not the limiting factor for pregnenolone formation, but rather the availability of cholesterol to CYP11A is what determines the rate of steroidogenesis.

Fig 1-2-1 illustrates ACTH causes the increase in intracellular cAMP which activates protein kinase, resulting in phosphorylation of a labile protein to act on cholesterol transportation (see details in section 1-3-1 & 1-4-1). In addition, ACTH also stimulates cholesterol ester hydrolysis in cytoplasmic lipid droplets, ensuring a continuous supply of substrate to activate steroidogenesis. Cycloheximide (CHX), a protein synthesis inhibitor, blocks the transfer of cholesterol to CYP11A without affecting transfer step 1 (DiBartolomeis and Jefcoate, 1984; Garren et al., 1971). Since cholesterol accumulates in the outer mitochondrial membrane, transfer step 2 is apparently prevented rather than transfer step 1 (Privalle et al., 1983), implying that the second step is more critical for steroidogenesis.

1.2.2 Biosynthetic pathways for steroid hormones

The biosynthesis of steroid hormones begins from the conversion of cholesterol to pregnenolone which is catalysed by CYP11A (Simpson and Boyd, 1967). The side-chain cleavage system consists of three components: a cytochrome P450 haemoprotein, a flavin adenine dinucleotide (FAD)-NADPH: adrenodoxin reductase, and an iron-sulphur protein (Simpson and Waterman, 1983). The steroidogenic

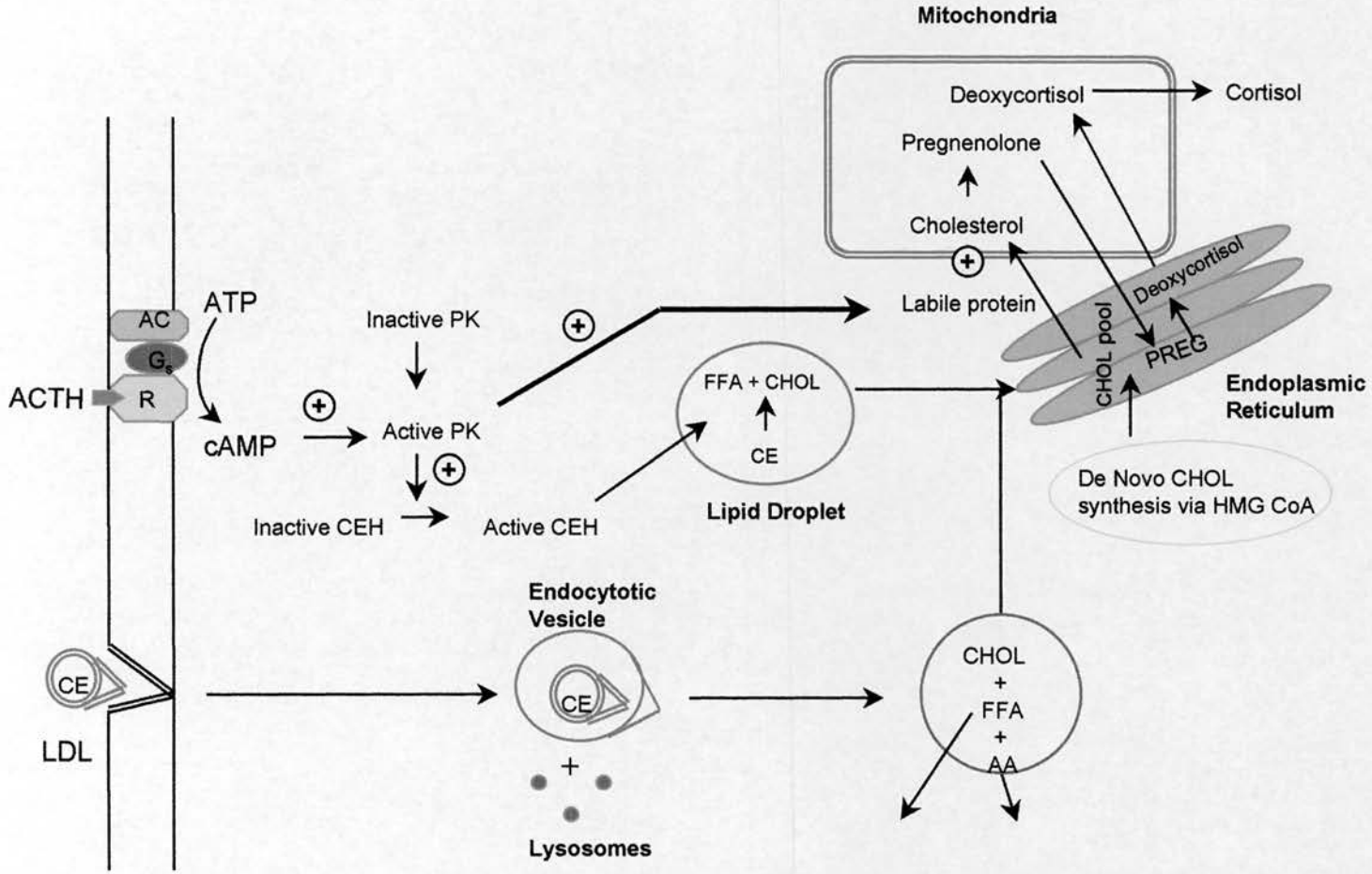

Fig. 1-2-1 Effects of ACTH on cholesterol metabolism in adrenal ZF cells. LDL: Low density lipoprotein; R: Receptor; G_s: G_s-protein; AC: Adenylate cyclase; CE: Cholesterol esters; CEH: Cholesterol ester hydrolase; CHOL: Cholesterol; FFA: Free fatty acids; AA: Amino acids; HMG CoA: 3-Hydroxy-3-methylglutaryl coenzyme; PK: Protein kinase. ⊕: Activation. PREG: Pregnenolone. Modified from Simpson and Waterman, 1983.

process is through a series of oxidation and reduction reactions catalysed by steroid dehydrogenases by several mitochondrial and microsomal steroid hydroxylases. Based on sequence homology between the various steroidogenic forms of cytochrome P450, CYP11A, cytochrome P450 17α-hydroxylase (CYP17) and cytochrome P450 21-hydroxylase (CYP21) are members of different gene families within the overall cytochrome P450 gene superfamily (Simpson and Waterman, 1988).

As shown in Fig. 1-2-1 and Fig. 1-2-2, in adrenal ZFR the conversion of cholesterol to pregnenolone occurs in the mitochondria. The major mechanism of this step is via an initial hydroxylation at the 22-position of cholesterol to yield (22R)-22-dihydroxycholesterol, then to pregnenolone. Pregenolone diffuses from mitochondria to endoplasmic reticulum/microsome and metabolised to 17α-hydroxypregneolone or alternatively to progesterone, then 17α-hydroxyprogesterone which is further metabolised to 11-deoxycortisol. The latter product is delivered back to mitochondria, subsequently for conversion to the end product, cortisol.

1.3 HORMONAL REGULATION OF STEROIDOGENESIS

Steroid hormone production in the adrenal gland is mainly regulated by trophic peptide hormones that stimulate steroidogenesis in their corresponding target tissues.

1.3.1 Intracelluar response to ACTH

1.3.1.1 The action of ACTH

ACTH, a principal regulatory factor of steroidogenesis (Haynes, 1958), is a major trophic hormone for the adrenocortical cells both *in vitro* and *in vivo*. ACTH regulates cholesterol metabolism through the control of cholesterol availability to CYP11A (Jefcoate et al., 1987).

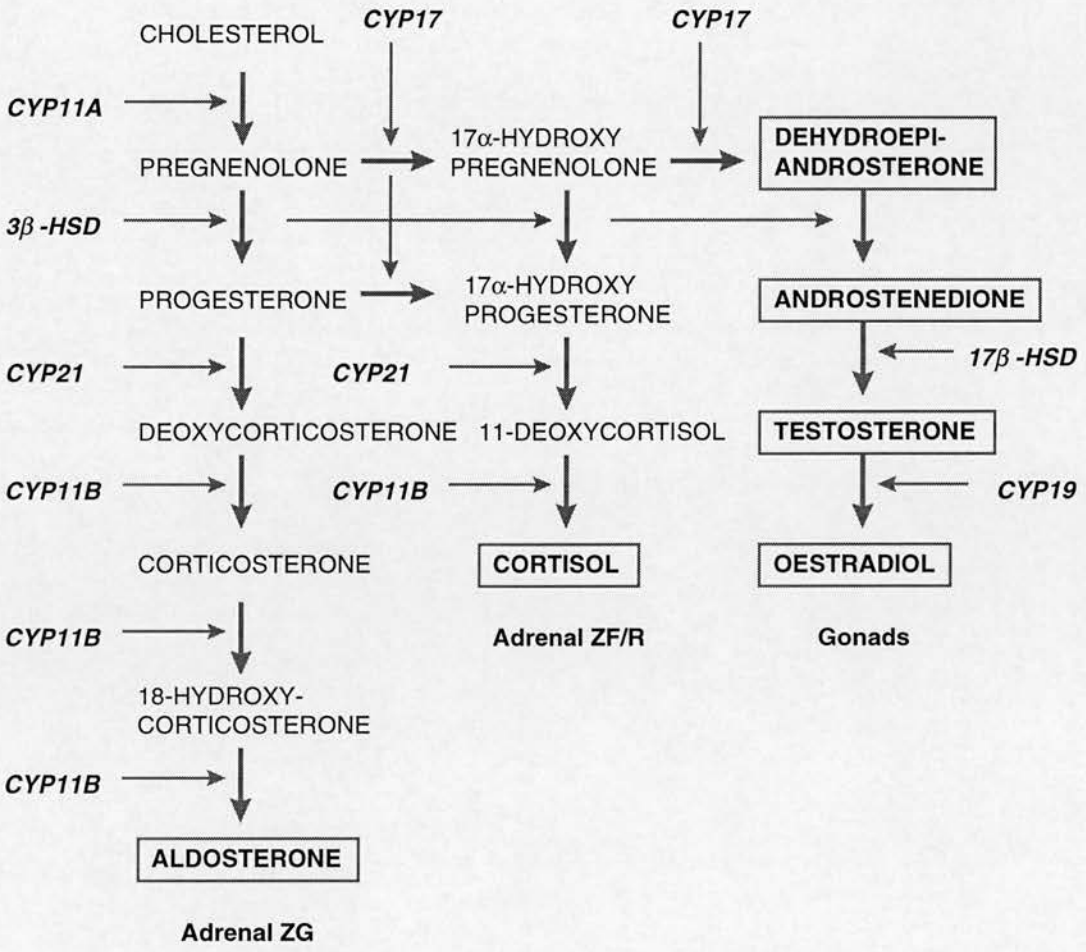

Fig. 1-2-2 Pathways for steroid hormone biosynthesis in bovine adrenal cortex and in gonads. CYP11A (P450scc): Cytochrome P450 side-chain cleavage enzyme; 3β-HSD: 3β-Hydroxysteroid dehydrogenease; CYP17: Cytochrome P450 17α-hydroxylase; CYP21: Cytochrome P450 21-hydroxylase; CYP11B: Aldosterone synthase (in adrenal ZG) or Cytochrome P450 11β-hydroxylase (in adrenal ZFR); 17β-HSD: 17β-hydroxysteroid dehydrogenase; CYP19: Cytochrome P450 aromatase. Modified from Brook and Marshall (Brook and Marshall, 1996).

ACTH is a 39-amino-acid peptide that stimulates secretion of glucocorticoids (corticosterone and cortisol), androgenic steroids and, to a lesser extent, mineralocorticoids. The first 24 NH_2-terminal amino acids of ACTH are the same in all species studied (Orth et al., 1992) and possess full biological activity in terms of corticosterone and cAMP production (Seelig and Sayers, 1973). On a molar basis, $ACTH_{1-24}$ and $ACTH_{1-39}$ are equipotent to replace bound [^{125}I]-ACTH and to stimulate cAMP and steroid production (Penhoat et al., 1989a). Synthetic $ACTH_{1-24}$ is therefore widely used for research and clinical purposes.

Fig. 1-3-1-1 illustrates that ACTH stimulates adrenal steroidogenesis by interacting with specific receptors (Schulster and Schwyzer, 1980) at the cell surface. ACTH receptors act through GTP-binding regulatory protein (G_s protein) to activate adenylate cyclase (Rodbell, 1980) and the further action of ACTH is mediated at least in part by protein kinase A (PKA) (Krueger and Orme-Johnson, 1983) which phosphorylates serine / threonine residues of proteins.

The acute effect of ACTH is to increase the conversion of cholesterol to pregnenolone. ACTH can maximally activate steroidogenesis with 5 min (Kim et al., 1997). The chronic effects of ACTH involve progressively increased synthesis of most enzymes of the steroidogenic pathway and more general actions on adrenocortical cell protein, RNA and DNA synthesis, and cell growth (Simpson and Waterman, 1983; Simpson and Waterman, 1988). Under chronic ACTH stimulation, the elevations of corticosteroids (corticosterone and aldosterone) and changes in mRNA and protein levels of steroidogenic enzymes in both adrenal ZG and ZFR were observed in rat adrenal *in vivo* (LeHoux et al., 1998).

1.3.1.2 ACTH receptor

ACTH receptor (ACTH-R) has a small number of high affinity sites for ACTH and a much larger number of low affinity sites. The existence of high- and low-affinity sites for ACTH in mouse adrenal tumour cells was proposed by Lefkowitz et al on the basis of [^{125}I]-ACTH binding studies. The data in Table 1-3-1 are a hypothetical projection of the number of receptors of each order occupied at various concentrations of ACTH (Lefkowitz et al., 1971).

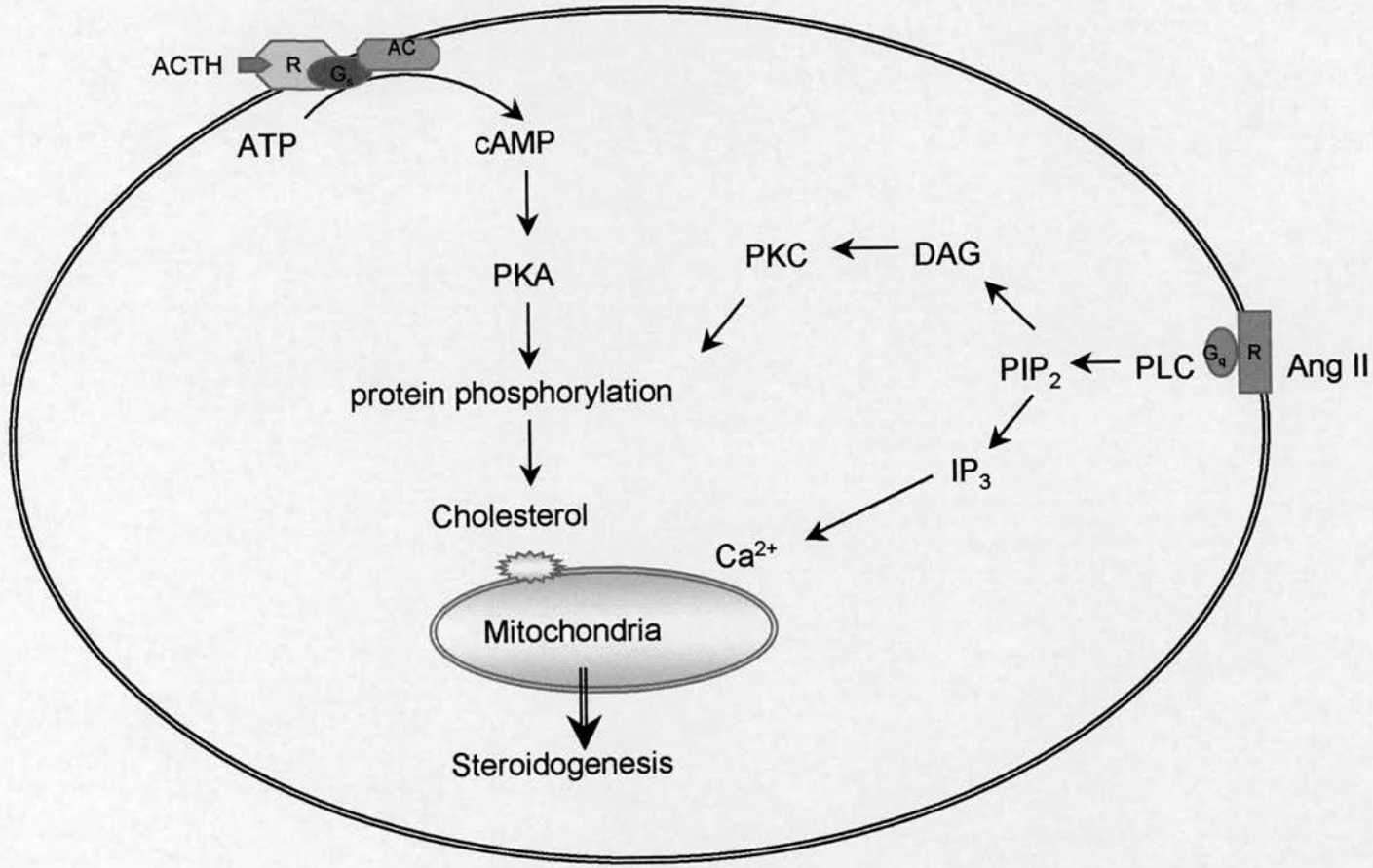

Fig. 1-3-1-1 Classic signalling pathways of steroidogenesis in response to trophic hormone stimulation in adrenocortical cells. R: Receptor; G: G-protein; AC: Adenylate cyclase; PKA: Protein kinase A; PLC: Phospholipase C; PIP_2: Phosphatidylinositol diphosphate; IP_3: Inositol 1, 4, 5- trisphosphate; DAG: Diacylglycerol; PKC: Protein kinase C.

Table 1-3-1 Relation of ACTH concentration to the number of receptors occupied

[ACTH] (M)	Sites occupied per cell	
	High affinity	Low affinity
10^{-14}	1	0
10^{-13}	5	1
10^{-12}	28	11
10^{-11}	54	108
10^{-10}	59	1080
10^{-9}	60	10,500
10^{-8}	60	83,000
10^{-7}	60	270,000
10^{-6}	60	348,000

Some investigators suggested that there may be two classes of ACTH receptors on adrenocortical cells (McIlhinney and Schulster, 1975). Two receptors or at least two different affinity sites may involved in the interaction of ACTH with rat adrenal cells (Moyle et al., 1973). Moreover, ACTH receptors on adrenocortical cells are much in excess of the those necessary for maximal steroidogenesis. The stimulation of a small fraction of receptors (<3%) is sufficient for maximal steroidogenesis (Buckley and Ramachandran, 1981).

Five transcripts of ACTH-R mRNA, two major of 1.8 and 3.4 kb and three minor of 4, 7 and 11 kb were found in the human adrenal (Lebrethon et al., 1994). Bovine adrenal ZFR cells express at least four ACTH-R transcripts, one major of 3.6 kb and three minor ones of 4.2, 1.8 and 1.3 kb. ACTH treatment increased the levels of ACTH-R mRNA in a time- and dose-dependent manner, i. e. ACTH has a positive effect on its own receptor, which is another important regulatory mechanism involved in the responsiveness of adrenocortical cells (Penhoat et al., 1994).

1.3.1.3 G-proteins and adenylate cyclases

The G-proteins, an extensive family, are the critical mediators between the receptors and a variety of effectors in the plasma membrane, consisting of three subunits. The largest one (39-46 kDa) has the α subunit which contains the binding site for guanine nucleotides. The second (β, 35-36 kDa) and third (γ, approx. 8 kDa) subunits form stable noncovalent complex, tightly coupling with the membrane. An interaction among multiple components allows a dynamic range of response to stimuli. The $\beta\gamma$ subunits are associated with a number of activities such as the activation of adenylate cyclase (AC) isoenzymes (Sternweis, 1996).

ACs are responsible for the conversion ATP to cyclic AMP. At least nine isoforms of AC have been identified and are classified into three groups: (i) Ca^{2+}/calmodulin-activated isoforms (type I, III and VIII); (ii) Ca^{2+}-inhibited (type V, VI and possibly IX) isoforms; (iii) Ca^{2+}-insensitive (type II, IV and VII) isoforms (Burnay et al, 1998; Taussig and Gilman, 1995) and more recently another isoform (sAC) was found in brain. The G-protein-coupled receptors can be differently integrated with AC depending on the properties and the level of isoforms in various tissues or cells (Defer et al., 2000).

1.3.2 Intracelluar response to angiotensin II (Ang II)

Ang II is a principal regulator of steroidogenesis in adrenal ZG, modulating aldosterone production. As shown in Fig. 1-3-1-1, Ang II binds angiotensin type 1 (AT1) receptors (Balla et al., 1991; Sasaki et al., 1991), which couple with phospholipase C (PLC) through $G_{q/11}$ protein. This action results in the hydrolysis of a plasma membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) generating diacylglycerol (DAG), an activator of protein kinase C (Hunyady et al., 1990; Lang and Vallotton, 1987) and inositol 1,4, 5-triphosphate (IP_3), which is responsible for calcium release from intracellular pools (Kojima et al., 1984; Rossier et al., 1987; Rossier, 1997).

Submitochondrial membrane analysis showed Ang II-stimulated exogenous cholesterol mobilisation to the mitochondria and a concomitant transport of

endogenous cholesterol to contact sites (Cherradi et al., 1996). Although well known as a major activator of aldosterone secretion by adrenal ZG cells, Ang II is also a potent effector of glucocorticoid secretion in bovine adrenal ZF cells *via* a distinct class of receptors (Hadjian et al., 1984). Several types of Ang II receptors have been identified in adrenal ZG and ZFR cells (Enyeart et al., 1996).

In addition to the PLC-DAG/IP3 second messenger system, Peytremann and co-workers firstly demonstrated that Ang II stimulated cAMP accumulation and subsequently increased steroidogenesis in bovine adrenal ZF cells (Peytremann et al., 1973). Ang II (10^{-7} M) caused a time-dependent increase in cAMP, reaching a 8-fold increase at 3 hr in fetal bovine adrenocortical cells (Bird et al., 1993).

Ang II has a potentiating effects on ACTH-stimulated cAMP in cultured bovine adrenal ZG cells (Baukal et al., 1994). There are at least two concepts with regard to the mechanism of Ang II-stimulated cAMP formation: (1) The mechanism of the combined effect of Ang II and ACTH may be that calcium entry into the cell plays an important role in the activation of adenylate cyclase (probably type III) by Ang II (Burnay et al., 1998); (2) the action of Ang II on cAMP formation may be through the cross-talk via PKC rather than that direct coupling with adenylate cyclase (Bird et al., 1993).

Apart from ACTH and Ang II, some other agents are also able to modulate the growth and function of the adrenal cortex, including insulin-like growth factor I (IGF-I) (Louveau et al., 1989; Penhoat et al., 1989b), atrial natriuretic factor (ANF) (Nawata et al., 1991), fibroblast growth factor (FGF) (Ehrhart-Bornstein et al., 1998), tumour necrosis factor-α (TNF-α) (Natarajan et al., 1989) and transforming growth factor-β (TGF-β) (Brand et al., 1998; Naville et al., 1991; Roy et al., 2000).

1.4 INTRACELLULAR SIGNALLING SYSTEMS RESPONDING TO TROPHIC HORMONES

Different intracellular signalling systems mediate the hormonal actions on steroid biosynthesis, depending on the type and intensity of the stimuli. Several major factors

have been proved to play a role in these signalling pathways. Amplification of the action of an initial stimulus is the central theme to signal transduction.

1.4.1 Cyclic adenosine 3', 5'-monophosphate (cAMP)

The role of cAMP as a second messenger was first revealed by Sutherland and co-workers in 1960s (Sutherland and Rall, 1960). cAMP is the primary intracellular mediator of ACTH-stimulated steroidogenesis in the adrenal gland (Mackie et al., 1972) and plays a key role in steroid synthesis in both the acute and chronic phase. It activates the hydrolysis of cholesterol esters in lipid droplets within seconds or minutes. In the chronic phase, cAMP regulates the transcriptional activation of genes encoding steroid hydroxylase and accessory proteins (Waterman, 1995).

ACTH induces an increase in intracellular cAMP via the activation of a G_s-protein-coupled to AC and consequently activates PKA and ultimately elevates steroid production.

Ang II can cause a small but significant increase in cAMP production via the AT1 receptors in bovine adrenal ZF cells. This result was demonstrated using four different radioimmunoassays for cAMP and three commercial sources of human synthetic Ang II. The cAMP production was specific and was both concentration- and time-dependent with a significant increase in the presence of 10^{-9} M Ang II and a maximal response at 10^{-7} M Ang II (Rainey et al., 1991). That Ang II can stimulate cAMP production has been demonstrated in a variety of tissues types. For example, there was a close correlation between Ang II-stimulated aldosterone production and cAMP levels in rat adrenal ZG cells (Bing and Schulster, 1978); Ang II also potentiated prostaglandin E_1 (PGE_1) stimulated cAMP accumulation in intact bovine adrenal medulla cells (Boarder et al., 1988).

A number of genes are stimulated by the elevation of cAMP and all include a responsive DNA element, TGACGTCA (Comb et al., 1986). Following the phosphorylation at Ser133 by cAMP-dependent protein kinase, cAMP response element binding (CREB) protein binds to CRE, regulating gene transcription (Gonzalez and Montminy, 1989). The mechanisms of this process are via the three

functional regions of CREB protein, i.e. (1) a transactivation domain with some potential phosphorylation sites; (2) a DNA binding domain; and (3) a "leucine zipper" dimerization domain (Gonzalez and Montminy, 1989; Hoeffler et al., 1988; Lali and Sassone-Corsi, 1994).

Apart from the activation of PKA, the later studies showed that cAMP mediates new signalling pathways in endocrine cells including the phosphorylation of protein kinase B (Richards, 2001). Cyclic AMP, once considered to be a straightforward second messenger, has been discovered to be a very complicated regulator. The action of this molecule is not only by stimulating protein phosphorylation through activation of protein kinase but also by inducing protein-protein interactions independently of any phosphorylation (Kawasaki et al., 1998).

1.4.2 Phospholipase A_2, arachidonic acid and its metabolites

Cellular phospholipids serve not only as the components of membrane structure, but also as the reservoirs of second messenger molecules that are generated by phospholipases. At least five types of phospholipases, i.e. A_1 (EC 3.1.1.32) , A_2 (EC 3.1.1.4), B (EC 3.1.1.5), C (two major types, EC 3.1.4.3) and D (EC 3.1.4.4) have been identified according to the position of their activity on the phospholipid backbone. Phospholipase A, C and D have all been proved to function both as enzymes and as signal transducers. The phospholipase A_2s (PLA_2s) are prominent members of the large group of signal-activated phospholipases (Balsinde et al., 1999; Rhee and Dennis, 1996).

1.4.2.1 Role of phospholipase A_2 in the arachidonic acid cascade

The phospholipase A_2 is a superfamily which consists of a broad range of enzymes defined by their ability to catalyse the hydrolysis of the middle (*sn*-2) ester bond of substrate phospholipids. Based on biological properties, these enzymes are considered as three main types, i.e. cytosolic PLA_2 ($cPLA_2$), intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$) and secretory PLA_2 ($sPLA_2$) (Balsinde et al., 1999).

Cytosolic PLA_2 ranging from 61 to 114 kDa is specific for arachidonic acid-carrying phospholipid, which is able to be translocated to membranes where its substrate is localised in response to increases in intracellular Ca^{2+}-(Dennis, 1994). It is believed to play an important role in the production of arachidonic acid metabolites, the eicosanoids. The essential role of $cPLA_2$ in AA metabolism has been proved using $cPLA_2$ knock-out mice (Bonventre et al., 1997). Intracellular Ca^{2+}-independent PLA_2 ranging from 84 to 90 kDa has been considered as a 'housekeeping enzyme' involved in the maintenance of membrane PL composition. Secretory PLA_2s are low molecular mass enzymes (13-18 kDa) that require Ca^{2+} to exert their action and do not exhibit preference for a fatty acyl chain in PL (Six and Dennis, 2000; Yedgar et al., 2000). More recently, PLA_2 have been classified into 11 Groups (I-XI) according to four criteria and each Group consists of several subgroups. cPLA2 falls into the category of Group IV, $iPLA_2$ into Group VI and $sPLA_2$ into Group IIA, IID, V and X (Murakami et al., 2000; Rhee and Dennis, 1996; Six and Dennis, 2000).

In intact cells, receptor-dependent activation of PLA_2 may occur via a G-protein-mediated mechanism (Axelrod et al., 1988; Gupta et al., 1990). The hydrolysis products of PLA_2 are free fatty acids such as arachidonic acid and oleic acid and lysophospholipid (Lyso-PL). They serve as the precursors for other lipid mediators and have also been considered as downstream effectors in signal transduction. The liberation of free AA is the rate-limiting step for the synthesis of eicosanoids which are key cellular mediators. The Lyso-PL can be converted to platelet-activating factor (PAF), another important lipid-derived mediator (Hirabayashi and Shimizu, 2000).

1.4.2.2 Routes leading to arachidonic acid release

Arachidonic acid (AA; 5, 8, 11, 14-eicosatetraenoic), a polyunsaturated fatty acid, is a ubiquitous precursor of biologically active eicosanoids and the liberation of AA from membrane phospholipids is a crucial step in signalling processes. Release of AA occurs via at least six routes, i.e. (i) cleavage of the glycerophospholipid (PL) backbone which is catalysed by various forms of PLA_2, yields free AA and Lyso-PL; (ii) activation of phospholipase C (PLC) forms DAG, which is subsequently hydrolysed by DAG-lipase into free AA and monoacylglycerol (MAG; Piomelli,1993);

(iii) activation of phospholipase A_1 (PLA_1) followed by lysophospholipase (phospholipase B) (Martin and Wysolmerski, 1987); (iv) activation of phospholipase D (PLD) followed by phosphatidate phosphohydrolase and DAG/MAG (Exton, 1990); (v) breakdown lipid esters from low density lipoproteins (LDL) (Habenicht et al., 1990); (vi) inhibition of the utilisation of lysophosphatidates in reacylation results in the increase in AA levels (Fuse et al., 1989). Under physiological conditions, the direct cleavage of AA from the *sn-2* position of phospholipids via PLA_2 is a key step for AA liberation (Balsinde et al., 1999).

1.4.2.3 Arachidonic acid and its metabolites

Arachidonic acid itself is associated with a number of cellular process, for example, the regulation of PKC, PLC, NADPH oxidase and Ca^{2+} oscillations (Hirabayashi and Shimizu, 2000). AA stimulates the conversion of cholesterol into pregnenolone in rat adrenal ZF cells (Mele et al., 1996).

More importantly, as shown in Fig.1-4-2-3, AA can be metabolised to diverse eicosanoids leading to more cellular responses via three pathways: (i) Cyclooxygenase (COX) catalyses the conversion of AA into two reactive intermediates, prostaglandin G (PGG) and prostaglandin H (PGH), which are precursors of the prostaglandins (PGs), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2); (ii) Lipoxygenase (LOX) forms hydroperoxyeicosatetraenoic acids (HPETE) as primary products, which can undergo a complex metabolism including reduction to corresponding hydroxyacids (HETE), or conversion into leukotrienes, epoxyhydroxides, etc; (iii) Cytochrome P-450 dependent monooxygenase (NADPH-P450 dependent epoxygenase) catalyses the conversion of AA into epoxyeicosatrienoic acids (EET), which can be hydrolysed to the corresponding dihydroxyeicosatetraenoic acids (diols/DHTs) by epoxide hydrolase (Piomelli, 1993).

Pharmacological and cell biology studies have demonstrated that COX-1, a constitutive isozyme, is limited to the acute phase of PG biosynthesis, which is elicited by Ca^{2+}-mobilizing agonists. On the other hand, COX-2, an inducible isozyme, is a prerequisite for the chronic response, which lasts for several hours following proinflammatory stimuli (Kuwata et al., 1998).

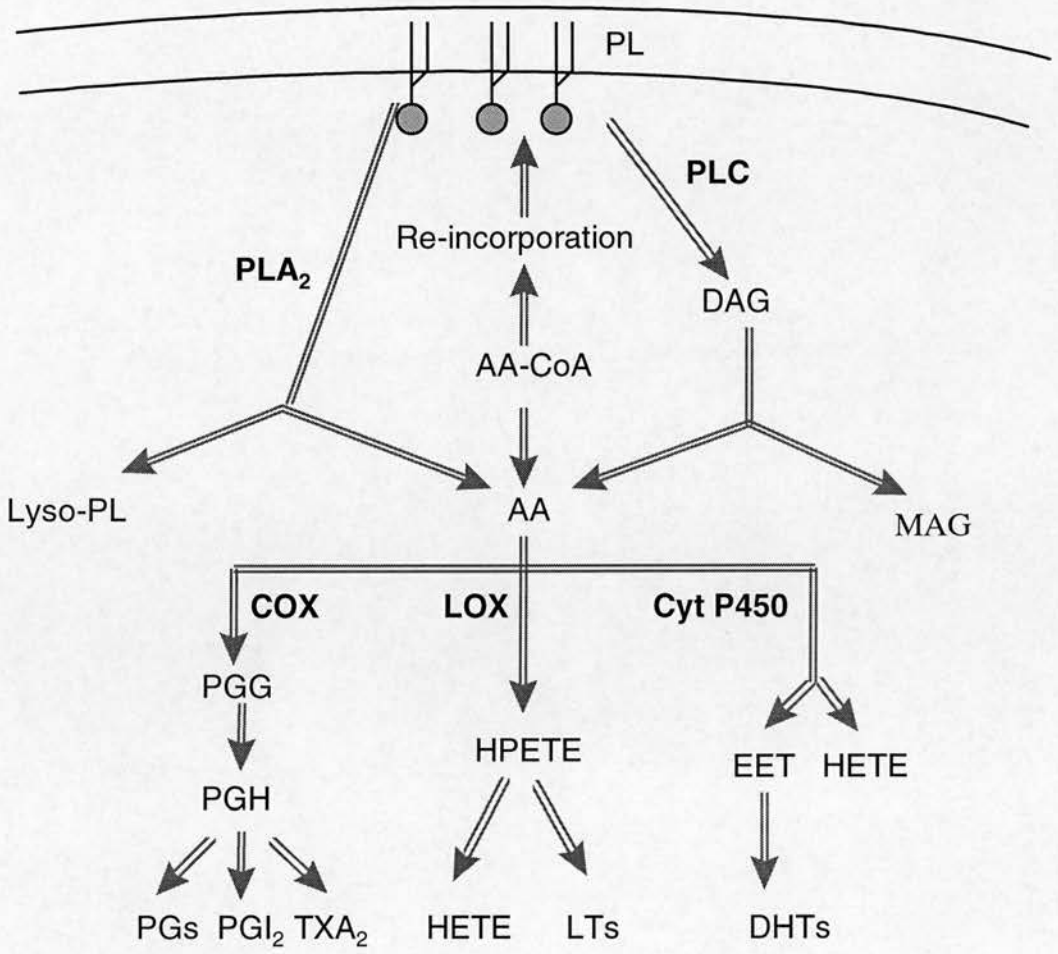

Fig. 1-4-2-3 Schematic overview of the cascade of arachidonic acid release (in part) and metabolism. PL: Glycerophospholipid; PLA_2: Phospholipase A_2; PLC: Phospholipase C; DAG: Diacylglyerol; MAG: Monoacylglycerol; AA: Arachidonic acid; AA-CoA: Arachidonoyl-coenzyme A; Lyso-PL; Lysophospholipid; Cyt P450: Cytochrome P450-dependent monooxygenase; LOX: Lipoxygenase; COX: Cyclooxygenase; HPETE: Hydroperoxyeicosatetraenoic acids; EET: Epoxyeicosatrienoic acid; HETE: Hydroxyeicosatetraenoic acids; PGG: Prostaglandin G; PGH: Prostaglandin H; PGs: Prostaglandins; PGI_2: Prostacyclin; TXA_2: Thromboxane A_2; LTs: Leukotrienes; DHTs: Dihydroxyeicosatetraenoic acids. Modified from Piomelli,1993.

Several LOX products of AA have been found to have potent biological activities and modulate physiological processes in various cells such as pancreatic β cells (Metz et al., 1983), pituitary cells (Snyder et al., 1983) and adrenocortical cells (Nishikawa et al., 1994).

Three types of LOX (5-, 12- and 15- LOX) have been identified in different tissues or species. The 5-LOX is present in rat adrenocortical cells. AA861, a 5-LOX inhibitor, reduced ACTH-stimulated corticosteroid secretion without changing the cAMP level, suggesting that the 5-HPETE, 5-LOX metabolite, may mediate steroidogenesis (Hirai et al., 1985). The 15-LOX metabolites have been shown to enhance steroidogenesis induced by as low as 10^{-12} M ACTH in bovine adrenocortical cells (Yamazaki et al., 1996). Bovine tracheal epithelial cells express a 12-LOX that has immunological reactivity similar to the leukocyte form and distinct from platelet 12-LOX; this LOX heterogeneity may provide a basis for their different roles (Hansbrough et al., 1990).

Cytochrome P450-dependent monoxygenase (MOX) pathway, the third route of AA metabolism, also performs important roles in cellular responses, presenting in the kidney (Morrison and Pascoe, 1981), liver (Oliw et al., 1982), pituitary gland (Capdevila et al., 1984), cornea (Schwartzman et al., 1987) and the adrenal cortex (Nishimura et al., 1989). This pathway is involved also in receptor-mediated signal transduction (McGiff, 1991).

In addition to generation of biologically active eicosanoids, AA can be reincorporated into phospholipids via reacylation of lysophosphatides by two enzymes (arachidonyl-CoA synthase and arachidonyl-CoA lysophosphatide acyltransferase) (Fuse et al., 1989). The reincorporation and various AA metabolites result in the AA cycle.

1.4.3 Calcium

Calcium (Ca^{2+}) is a ubiquitous messenger with both versatility and universality and has an important role in controlling cellular responses. Cells in their resting state have free Ca^{2+} concentrations of 100 nM but are activated when the level of Ca^{2+} rises to approximately 1000 nM. Ca^{2+} signals often present in a temporal pattern such as Ca^{2+} spikes (Berridge et al., 2000).

There exist many possible routes of Ca^{2+} participation in the regulation of metabolism. Ca^{2+} can act as both a primary and secondary messenger in mediating many essential cellular functions. Several Ca^{2+}-selective channels have been identified, i.e. voltage-, extracellular ligand-, intracellular messenger- and mechanically-operated channels. For each channel there are several subtypes, for example, voltage-operated Ca^{2+} channel includes L-, T-, N-, P-, Q- and R- type (Rossier, 1997).

A requirement for Ca^{2+} in steroidogenesis of the adrenal cortex is well established. Ca^{2+} is a major second messenger for NPS-ACTH-stimulated steroidogenesis in bovine adrenal ZFR cells (Yamazaki et al., 1998). ACTH at 10^{-12} M causes a transient increase in cytoplasmic free calcium concentration ($[Ca^{2+}]_c$) by a cAMP-independent mechanism and this effect appears to be saturated at 10^{-10} M ACTH in bovine ZG cells (Kojima and Ogata, 1986). In bovine adrenal ZF cells, the addition of ACTH (10^{-13}-10^{-10} M) triggers oscillations or sustained elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) (Kimoto et al., 1996). The increase in $[Ca^{2+}]_i$ may result in the rise in intramitochondrial Ca^{2+} concentration, which can induce the acute steroidogenic response possibly due to the activation of mitochondrial enzymes involved in NAD(P)H production in bovine adrenal ZF cells (Hoolahan et al., 2000).

Enyeart et al proposed Ca^{2+} as a primary intracellular messenger regulating steroidogenesis, particularly at picomolar ACTH concentrations (Enyeart et al., 1993). More recently, some results suggest that capacitative calcium entry (i.e. Ca^{2+} entry triggered by depletion of intracellular Ca^{2+} stores) is involved in steroidogenesis in bovine adrenal ZF cells (Ebisawa et al., 2000).

Python and co-workers showed that Ca^{2+} levels regulated the production of both pregnenolone and aldosterone in bovine ZG cells. The formation of pregnenolone from freely diffusible analogues of cholesterol was not affected by Ca^{2+}. This is suggestive that the potential target of Ca^{2+} occurs at the very early steps of steroidogenesis prior to cleavage of cholesterol (Python et al., 1995).

The mechanism of Ca^{2+} on steroidogenesis was further studied by Cherradi et al who demonstrated that one of the main functions of the Ca^{2+} messenger is to

increase cholesterol supply to CYP11A by enhancing intermembrane cholesterol transfer. Studies using submitochondrial fractions revealed that stimulation of bovine adrenal ZG cells with Ca^{2+} led to a decrease in cholesterol content at the outer membrane and a concomitant increase in cholesterol at both contact site and inner membrane (Cherradi et al., 1996).

Furthermore, Ca^{2+} also mediates steroidogenesis induced by other stimuli. A rapid rise in cytosolic free calcium triggers aldosterone production in response to Ang II and potassium in bovine adrenal ZG cells (Brandenburger et al., 1996; Capponi et al., 1984). Ang II enhances aldosterone biosynthesis through the stimulation of two distinct Ca^{2+} entry pathways: (1) Opening the voltage-operated calcium channel (VOC); and (2) activation of a capacitative Ca^{2+} entry (Burnay et al., 1998). The changes in cytosolic calcium and aldosterone stimulated by Ang II show a complex dose-response pattern in rat adrenal ZG cells, basically including a sustained Ca^{2+} response and an oscillating Ca^{2+} response (Quinn et al., 1991).

1.*4.4 Interaction between signalling pathways*

The signalling network is composed of multiple components. It has became increasingly important to understand that a signalling pathway not only works in a linear fashion but also interacts with other pathways, resulting in a complex network. Interaction of signal transduction systems leads to substantial amplification of the original signal at each stage of the cascade, ensuring not only efficient stimulation of steroid biosynthesis but possibly also efficient switching off the initial steroidogenic stimulus.

Ca^{2+} is known to interact with other intracellular messenger systems such as cAMP. Ca^{2+}- and PKC-mediated regulation of cAMP production has been documented in several studies (Cooper et al., 1995; Morimoto and Koshland, 1994; Warhurst et al., 1994). The changes of cAMP can affect the levels of Ca^{2+} by acting on both Ca^{2+} channels and pumps (Berridge et al., 2000). In the bovine adrenal ZF cells, ACTH-induced cAMP increase may be potentiated by Ang II through a capacitative calcium influx (Baukal et al., 1994).

Analysis of the CYP11B2 promoter in H295R cells showed that the cAMP-, K^+- and Ang II responsive regions localise to a common responsive element, implying a potential cross-talk among multiple signalling pathways (Clyne et al., 1997). Another interaction may be through the AA cascade. AA and/or its LOX products modulate K^+ channels (Kim and Clapham, 1989; Ordway et al., 1989). The AA metabolites produced by cytochrome P450 monooxygenase also inhibited Na^+/K^+ ATPase activity (McGiff, 1991).

Signal networking results in several emergent properties that the individual pathways do not have, including extended signal duration, activation of feedback loops, definition of threshold stimulation for biological effects and multiple signal outputs (Bhalla and Iyengar, 1999). At present, the studies on signal transduction can also be conducted by computational methods, providing a more comprehensive scenario in this field.

1.5 PROTEIN KINASE SYSTEMS

The phosphorylation and dephosphorylation of proteins are important regulatory mechanisms in response to a number of stimuli, modulating the protein functions in a rapid and reversible manner. Protein phosphorylation is particularly prominent for signal transduction (Edelman et al., 1987).

1.5.1 Protein kinase A

Protein kinase (PKA)-dependent phosphorylation is a key intermediate step in the action of ACTH (Wong et al., 1992). PKA exists as a heterotetramer, consisting of a dimer of regulatory subunits (R) and two monomeric catalytic subunits (C) (Francis and Corbin, 1994). Cyclic AMP activates PKA which phosphorylates serine (Ser) / threonine (Thr) residues in target proteins containing the consensus sequence Arg-Arg-X-Ser/Thr-X (Arg: Arginine; X: any amino acid) (Kennelly and Krebs, 1991).

1.5.2 Ca^{2+}/calmodulin-dependent kinase II

Many intracellular effects of Ca^{2+} are mediated by Ca^{2+}/calmodulin-dependent (CaM) kinase II. CaM kinase II is a complex of 12 subunits that exists in four differentially-expressed forms (α, β, γ and δ). In the inactive state there is a strong interaction between the inhibitory and catalytic domains of the enzyme. The binding of Ca^{2+}/calmodulin allows the catalytic domain to phosphorylate the inhibitory domain. Once activated, CaM kinase II retains significant activity even after the removal of Ca^{2+}, thereby prolonging the duration of kinase activity (Quadroni et al., 1998; Schulman, 1988; Siekevitz, 1991).

1.5.3 Protein kinase C

Protein kinase C, a serine/threonine kinase, is composed of a single polypeptide chain of 77-83 kDa, containing the regulatory and the catalytic domain (Azzi et al., 1992) and classified into 11 isotypes on the basis of structural and functional characteristics (Parker, 1996). The PKC isozymes act differentially according to the properties of their activators, and fall into three groups (1) Calcium- and phospholipid-dependent PKC (α, β_I, β_{II} and γ); (2) calcium-independent and phospholipid-dependent PKC (δ, ε, η, θ and μ); and (3) calcium- and phospholipid-independent PKC (λ/ι and ξ) (Zoukhri et al., 1997).

PKC can be activated by a number of agents including Ca^{2+}, lipid (fatty acids), and phorbol ester, etc. PKC is directly activated by the tumour promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA) and presumably acts as a binding site and cellular mediator of TPA (Kikkawa et al., 1984). The computational analysis based on the experimental concentration-effective curves has demonstrated that PKC is activated by Ca^{2+}, AA and DAG synergistically (Bhalla and Iyengar, 1999; Schaechter and Benowitz, 1993; Shinomura et al., 1991).

The involvement of protein kinases in the signalling pathways controlling steroidogenesis has been well established. Furthermore, the dephosphorylation by phosphoprotein phosphatase is also an important regulatory mechanism for steroidogenesis (Jones et al., 2000; Sayed et al., 1997).

1.6 CANDIDATE PROTEINS FOR REGULATION OF STEROIDOGENESIS

Steroid hormone synthesis is regulated via controlling cholesterol delivery to the inner mitochondrial membrane. The diffusion of the hydrophobic cholesterol through the aqueous layer between the outer and the inner mitochondrial membrane is extremely slow (Phillips et al., 1987), the transfer of cholesterol must therefore occur in an assisted manner (Stocco, 1997), i.e. this process is mediated by an acute regulator or regulators. In early 1960s, Ferguson discovered a puromycin-sensitive protein which could be a key factor for increasing steroid output (Ferguson, 1962 and 1963). The studies using protein synthesis inhibitors (puromycin and cycloheximide) showed both inhibitors blocked ACTH-induced steroid production in the adrenal cortex, which may be caused by the inhibition of the synthesis of a rapidly turning-over protein (Garren et al., 1965). Cycloheximide treatment had resulted in cholesterol accumulation in the outer mitochondrial membrane but had no direct effect on the activity of CYP11A itself (Privalle et al., 1987). Furthermore, acute steroidogenesis evidently excludes the involvement of newly synthesised CYP11A as the change in this steroidogenic enzyme was only significant after 6-12 hr ACTH treatment (Simpson and Waterman, 1983). This implied that an action prior to the first steroidogenic reaction was a critical step. Thus, a number of experiments have been carried out to search for a "labile protein" required for cholesterol transport from the outer to the inner mitochondrial membrane. Several candidate proteins have been proposed.

1.6.1 Sterol carrier protein

Sterol carrier protein (SCP_2), a 13 kDa protein, may be related to delivery of cholesterol from lipid droplet stores into mitochondria for initiation of steroid hormone synthesis (Chanderbhan et al., 1982; Vahouny et al., 1983). However, SCP_2 is not affected by cycloheximide treatment and not acutely regulated by hormone (van Amerongen et al., 1989). Moreover, SCP_2 is non-specific lipid transfer protein because it promotes the transfer of a variety of lipids including sterols, phospholipids and glycolipids (Kallen et al., 1998b).

1.6.2 Steroidogenesis activator polypeptide

Steroidogenesis activator polypeptide (SAP), a 3.2 kDa small molecular (the precursor is 78 kDa glucose-regulated protein, GRP78) exhibits cycloheximide sensitivity that is the characteristic of ACTH-induced labile activator for adrenal steroidogenesis (Li et al., 1989; Mertz and Pedersen, 1989; Pedersen and Brownie, 1983; Pedersen and Brownie, 1987).

1.6.3 Peripheral benzodiazepine receptor

Peripheral benzodiazepine receptor (PBR), a 18 kDa protein, is primarily located on the outer mitochondrial membrane of adrenal gland and is involved in cholesterol transfer from the outer to the inner mitochondrial membrane for steroidogenesis (Anholt et al., 1986; Krueger and Papadopoulos, 1990; Papadopoulos, 1993). A 3-dimensional protein model indicates that PBR contains five transmembrane domains, forming a channel-like structure that can accommodate a cholesterol molecule (Bernassau et al., 1993). The ability of PBR to bind benzodiapines is associated with a 34 kDa voltage-dependent anion channel, suggesting that PBR is a multimeric complex (Garnier et al., 1994). PBR may serve as a channel by which cholesterol can freely enter mitochondria and PBR ligands may control the opening of this channel, i.e. cholesterol is captured by PBR and released upon ligand binding (Amri et al., 1998).

1.6.4 Steroidogenic acute regulatory protein

Some work has demonstrated that inhibition of protein synthesis had no effect on the increased delivery rate of cholesterol from cellular stores to the outer mitochondrial membrane, but that the subsequent delivery from the outer to the inner mitochondrial membrane was completely inhibited by cycloheximide (Privalle et al., 1983; Privalle et al., 1987). The characteristic of cycloheximide sensitivity implies that the labile protein for cholesterol transport is *de novo* synthesised in response to trophic hormone treatment. Initially the investigation using 2-dimensional gel electrophoresis revealed protein i produced in response to ACTH, differing from protein p in unstimulated rat adrenal ZFR cells (Krueger and Orme-Johnson, 1983). Further

studies demonstrated that steroidogenesis was regulated by ACTH-induced 28, 30, 32 and 37 kDa phosphoproteins with various pI values (Epstein and Orme-Johnson, 1991; Pon et al., 1986; Pon and Orme-Johnson, 1986). The direct evidence of a cause and effect relationship between the expression of a 30 kDa protein and an increase in steroidogenesis was established in transient transfection of MA-10 Leydig tumor cells by Clark and co-workers in 1994. They concluded that this protein is required in the acute regulation of steroidogenesis and proposed to call it **St**eroidogenic **A**cute **R**egulatory protein, later abbreviated to StAR protein (Clark et al., 1994). More importantly, congenital lipoid adrenal hyperplasia (CAH), a steroid deficiency disease (i.e. a human gene knockout for *StAR* gene) has demonstrated that truncated and non-functional StAR protein leads to steroidogenic disorder (Lin et al., 1995). In contrast, other candidates (SCP2, GRP78 and PBR) are normal in this clinical condition (Lin et al., 1993; Lin et al., 1991). This discovery provided compelling evidence for the function of StAR in steroidogenesis on a genetic basis. Furthermore, an essential role for StAR protein in adrenocortical and gonadal steroidogenesis has also been demonstrated by the experimental model for StAR knockout in mice (Caron et al., 1997).

1.6.5 Arachidonic acid-related thioesterase involved in steroidogenesis

A novel phosphoprotein protein (43 kDa) named as arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt) also participates in the control of acute steroid synthesis and was found in adrenal, ovary placenta and brain (Cymeryng et al, 1995; Finkielstein et al, 1998; Maloberti et al, 2000).

1.7 StAR PROTEIN

Since StAR protein was established as meeting many of the expected criteria for a labile regulatory protein that facilitates cholesterol delivery for initiation of the steroidogenic pathway, intensive investigations have recently been conducted in this field.

1.7.1 Further characteristics of StAR protein

StAR protein may be a transporter that binds and transfers cholesterol through the mitochondrial intermembrane space thereby playing a key role in steroid hormone synthesis.

1.7.1.1 Organisation of StAR gene structure and protein

The *StAR* locus was detected in chromosome 8p11.2 by fluorescence *in situ* hybridisation and a *StAR* pseudogene was mapped to chromosome 13 in the human (Sugawara et al., 1995a). StAR gene spans 8 kb and consists of seven exons interrupted by six introns ranging from 141 nt to 2 kb (Sugawara et al., 1995b). Fig. 1-7-1-1a illustrates that the mouse *StAR* gene is rather compact and contains seven exons and six introns consisting totally of 6.5 kb (Clark et al., 1995).

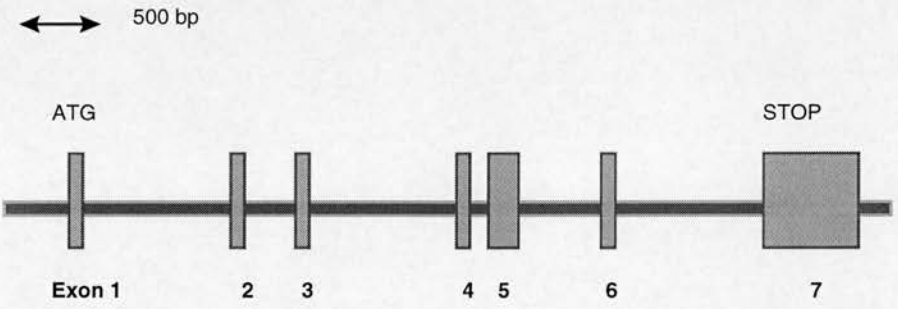

Fig. 1-7-1-1a Schematic representation of mouse StAR gene structure

StAR protein sequence is highly conserved with over 80% homology and 90% similarity across mouse (Clark et al., 1994), human (Sugawara et al., 1995) and rat (Lee et al., 1997; Mizutani et al., 1997).

The bovine StAR cDNA encodes a 285 amino acid protein (Fig.1-7-1-1b) (Hartung et al., 1995) and its DNA and protein sequence accession number are EMBL: BTY 17259 2 and SWISS-PROT:Q28918 respectively.

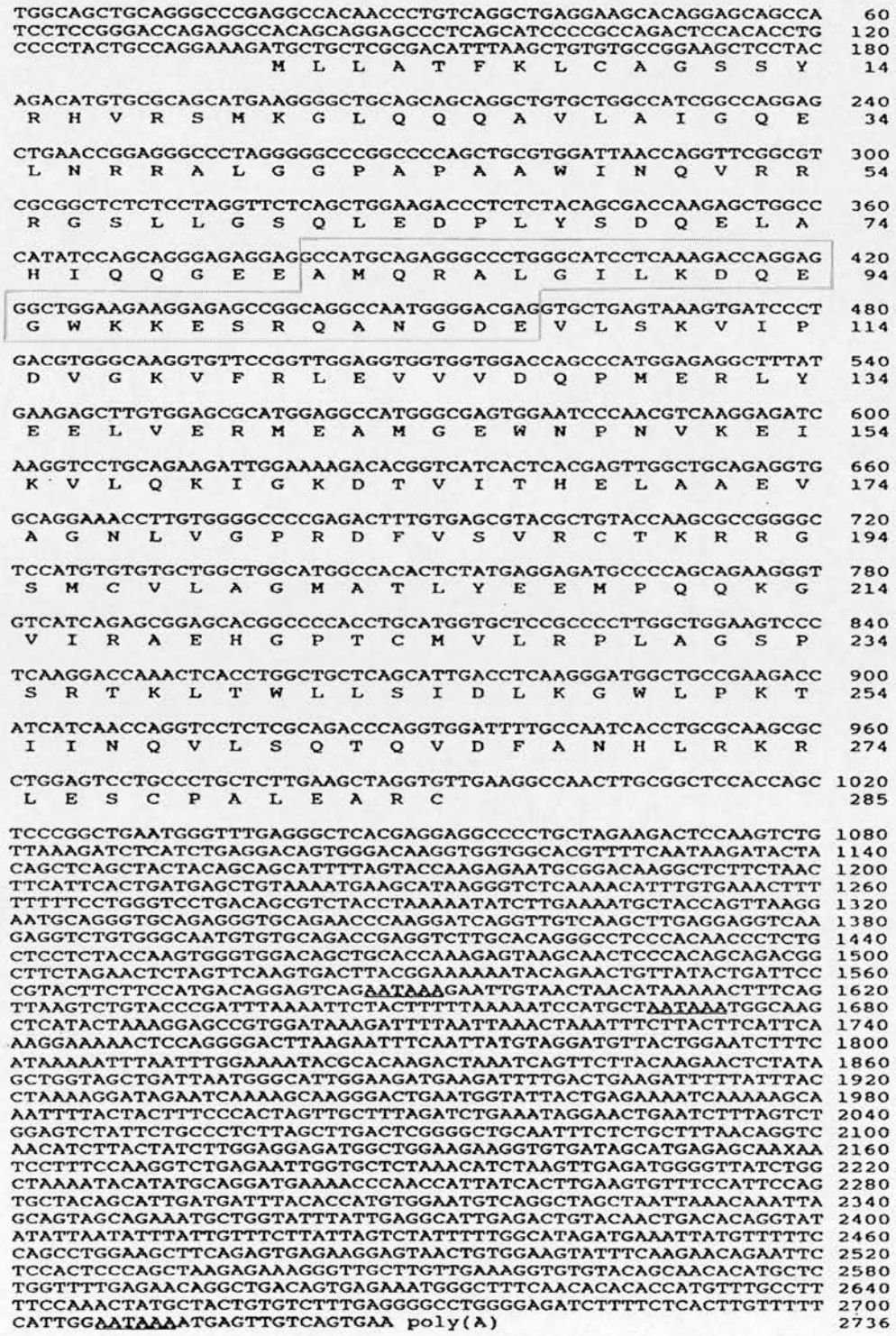

Fig. 1-7-1-1b Complete nucleotide sequence of bovine StAR gene transcript and amino acid sequence of StAR protein. The position of peptide StAR antigen is indicated in the box (Adapted from Hartung et al, 1995).

1.7.1.2 Distribution of StAR

The expression of *StAR* is predominantly restricted to organs that carry out mitochondrial sterol hydroxylation reactions, which are under acute regulation by tropic hormones. Immunoblotting and *in situ* hybridisation analysis demonstrated that StAR protein was specifically expressed in the adrenal cortex, ovary and testis of adult mice (Clark et al., 1995). Low levels of StAR transcripts in rat brain were observed using RT-PCR (Furukawa et al., 1998) which is more sensitive than the analysis by Northern blot analysis. *StAR* was also found expressed in porcine and bovine placenta (Pilon et al., 1997). As summarised in Table 1.7.1.2, two StAR mRNA species (1.6 and 3.5 kb) were found in rat adrenal and the amount of the larger mRNA species was consistently much higher than that of the small size (Ariyoshi et al., 1998). Two major transcripts (3.4 and 1.6 kb) and a minor one (2.7 kb) were revealed in MA-10 Leydig tumor cells (Clark et al., 1995). A major StAR transcript of 1.6 kb and less abundant transcripts of 4.4 and 7.5 kb were detected in human ovary and testis, but not in placenta or the urogenital system (Gradi et al., 1995; Sugawara et al., 1995a). The *StAR* gene is transcribed as 3.0 and 1.8 kb in bovine ovarian corpora lutea (Hartung et al., 1995), and a minor transcript (1.3 kb) was observed later (Bao et al., 1998). The ovine StAR cDNA hybridised to a major band (2.8 kb) and a minor band (1.6 kb) in ovine corpora lutea and in the adrenal gland (Juengel et al., 1995). Two major transcripts of 1.7 and 2.7 kb of StAR was detected in H295R cells (Clark and Combs, 1999).

Table 1-7-1-2 StAR transcripts in various species

Species and tissues	Transcripts (kb)	Reference
Rat adrenal	3.5 and 1.6	(Ariyoshi et al., 1998)
MA-10 cells	3.4, 1.6 and 2.7	(Clark et al., 1995)
Human ovary and testis	1.6, 4.4 and 7.4	(Sugawara et al., 1995).
Bovine ovary	3.0 and 1.8	(Hartung et al., 1995)
Ovine adrenal	2.8 and 1.6	(Juengel et al., 1995)
H295R cell	1.7 and 2.7	(Clark and Combs, 1999)

Apart from adrenal and gonadal glands, the presence of StAR protein in other organs (e.g. kidney and placenta) appears variable among different species, which may be due to species specificity. However, the possibility that this variation could be caused by the sensitivity of detection methods employed can not be ruled out. More recently, StAR transcripts were also found in lower vertebrates, for example in the ovary, testis, kidney and brain of zebrafish (Bauer et al., 2000).

1.7.1.3 Various forms of StAR protein

StAR appears as a family of proteins. A set of four protein species in rat could be produced from a core protein and combination of two different modifications (Epstein and Orme-Johnson, 1991). The 2-D data show two sets of labelled protein (four of 28.5 kDa and four of 30 kDa) in terms of molecular mass and isoelectric points. The set of smaller proteins 1-4 (pI=6.90, 6.59, 6.44, 6.33) exhibit a systematic relationship to the larger proteins 5-8 (pI=7.05, 6.67, 6.53, 6.43). Each smaller protein has a pI that is approximately a value of 0.1 lower than the corresponding larger proteins in bovine ZG cells and similar results were obtained from bovine fasciculata cells (Elliott et al., 1993, Elliott, 1997). Jefcoate's group also demonstrated that a set of eight proteins were formed after modification in response to cAMP in the bovine adrenal (Kim et al., 1997). StAR is synthesised as a 37 kDa precursor, imported into the mitochondria, processed to its 30 kDa mature forms, and localised to the intermembrane space (King et al., 1995).

1.7.1.4 Phosphorylation of StAR protein

StAR is a phosphorylated protein (Epstein and Orme-Johnson, 1991; Hartigan et al., 1995), it contains three PKA/calmodulin-dependent kinase II and one PKC phosphorylation sites. Two consensus sequences for phosphorylation by PKA are conserved across all species in which StAR protein has been sequenced. Serine 195 (human StAR amino acid sequence) is important for biological activity of StAR protein. Serine 277 exists in bovine, human and rat but not in mouse (Arakane et al., 1997; Ariyoshi et al., 1998).

1. 7.1.5 Up- and down-regulation of StAR protein

The expression of StAR protein is acutely up-regulated by trophic hormones. Besides ACTH and Ang II, other trophic hormones such as luteinizing hormone (LH) also elevated StAR mRNA and protein in bovine ovary (Ivell et al., 2000). LH and/or growth hormone (GH) increased amounts of StAR mRNA in ovine corpora lutea (Juengel et al., 1995). Follicle-stimulating hormone (FSH) increased StAR mRNA levels in porcine granulosa cells (Balasubramanian et al., 1997; Pescador et al., 1997). Both insulin and insulin-like growth factors enhanced the StAR gene expression in bovine luteinized granulosa cells (Mamluk et al., 1999).

In contrast, a hormone such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a luteolytic agent, caused a decrease in StAR mRNA in human and ovine corpora lutea (Chung et al., 1998; Juengel et al., 1995). $PGF_{2\alpha}$ induced a rapid decline in progesterone production and StAR protein expression in isolated rat corpus luteum without altering mRNA expression (Fiedler et al., 1999). StAR protein gene transcription is decreased by atrial natriuretic peptide (ANP) in bovine adrenal ZG cells (Cherradi et al., 1998b). Tumour necrosis factor-α (TNFα) reduces porcine Leydig cell testosterone secretion induced by LH/hCG (human chorigonadotropin) mainly through the inhibition of StAR protein expression (Mauduit et al., 1998). Transforming growth factors-βs (TGF-βs) decrease cholesterol supply to mitochondria via repression of StAR, which occurs at the transcription level (Brand et al., 1998). The negative regulatory role of hormones may be a balance mechanism, in contrast to the major positive agents such as ACTH, LH and FSH, which controls overall endocrine functions of the adrenal and the gonads.

1.7.1.6 Models of the mechanisms for StAR protein action

Although the precise mechanism of StAR's action in transferring cholesterol to the inner mitochondrial membrane is not well understood, several models have been proposed to reflect the possible machinery.

1) Model A: StAR import to mitochondria is obligatory for stimulation of steroidogenesis (Stocco and Clark, 1996). The observation that StAR is localised

to contact sites between the outer and inner mitochondrial membrane (Cherradi et al., 1997) is likely to support the hypothesis for the mitochondrial import mechanism of StAR protein.

2) Model B: StAR protein acts on the outside of mitochondria to stimulate steroidogenesis (Arakane et al., 1998). This model was based on: NH_2-terminal deletion mutants of StAR had steroidogenic activity equivalent to wild-type StAR although the mutants could not enter the mitochondria (Arakane et al., 1996) and His-tag StAR lacking the mitochondrial targeting sequence stimulated pregeneolone synthesis to the same extent as wild-type StAR. The C-terminal region of StAR protein is extremely important in cholesterol transfer (Arakane et al., 1998). The observation on the effect of wild-type StAR and several modified forms of StAR on intramitochondrial cholesterol transfer also suggested that StAR did not enter the mitochondria to facilitate cholesterol transfer (Wang et al., 1998). Furthermore, the recombinant StAR protein expressed in COS-1 cells indicated that StAR protein retained activity even without entry into the inner mitochondria (Kallen et al., 1998b).

3) Model C: The translocation of cholesterol from the outer to inner mitochondrial membrane is a putative transfer action, a process that involves probably several mediators and particularly StAR protein, which may rely on the complex of StAR, PBR, SAP and perhaps other as yet unidentified protein (Stocco, 1999). StAR protein might act as a molecular ferry or it could activate a ferry system which transports the cholesterol through PBR from the outer to inner mitochondrial membrane (Thomson, 1998). Furthermore, StAR protein probably plays a role in forming a channel for cholesterol entry by interacting with a protein on the outer mitochondrial membrane (e.g. PBR). PBR is an absolute requirement for determining the ability of uptake cholesterol, whereas StAR protein may offer a continuous supply (Amri et al., 1998). More recently, the study on fluorescence energy transfer between proteins using green fluorescent protein (GFP, donor fluorophore) and yellow fluorescent protein (YFP, acceptor flourophore) has demonstrated that StAR protein and PBR are associated in the process of cholesterol delivery (West et al., 2001).

4) Model D: The action of StAR protein is a transient and subtle process. It was proposed that StAR is only active immediately after cleavage of the matrix targeting sequence; StAR functions at very low concentrations, and the targeting sequence of StAR facilitates this high affinity process through interactions within the mitochondrial matrix (Artemenko et al., 2000).

5) Model E: Several recent studies have begun to shed light on the mechanisms of the precise structure and function of StAR protein. The comparison between wild-type and CAH mutant StAR structures indicates differences in protein folding. The wild-type and a partially active mutant are predominantly α-helical with some intramolecular β-sheets. While, the inactive mutants that lost charged residues retain much of their α-helical and tend to form intermolecular β-sheet. This is suggestive that mutant StAR proteins that cause lipoid CAH are probably due to protein misfolding (Bose et al., 1998). The active form of StAR protein appears to be a molten globule. The molten globule state would provide an efficient pathway for minimising the energetic cost, a compact protein is therefore easily insert into a membrane. StAR may undergo a conformational shift to an extended structure that interacts with the outer mitochondrial membrane, permitting this molten globule to act as an on/off switch (Bose et al., 1999).

6) Model F: StAR protein contains a StAR-related lipid transfer (START) domain which is a 200-210 amino acid motif that appears to be a common feature for a remarkably wide range of proteins involved in diverse functions such as lipid transport and metabolism. The START domain structure is a predominantly hydrophobic tunnel which is 26Å x 12Å x 11Å in size extending almost the entire of the protein and is likely to be large enough to hold a single cholesterol molecule Both StAR and MLN64 START domains can bind 1 mole cholesterol per mole of protein *in vitro*. This binding stoichiometry implies that StAR protein may act by shuttling one cholesterol molecule at a time across the mitochondrial intermembrane space (Ponting and Aravind, 1999; Tsujishita and Hurley, 2000). The START domain suggests that Serine 195 does not play any part in the overall conformational regulation nor a direct role in cholesterol binding, implying that phosphorylation of this domain may involve the N-terminal mitochondrial targeting, rather than the ability of binding cholesterol (Tsujishita and Hurley, 2000).

1.7.1.7 Other possible functions of StAR protein

The actions of StAR protein are not only specific to steroidogenesis but also extend to other mitochondrial cholesterol-metabolising enzymes. StAR protein increases the formation of 3β-hydroxy-5-cholestenoic acid via stimulating mitochondrial cholesterol 27-hydroxylase activity in transfected COS-1 cells (Sugawara et al., 1995b). StAR transcripts have been also found in the human kidney, in which StAR increases 1α-hydroxylation of vitamin D in response to parathyroid hormone (Gradi et al., 1995; Sugawara et al., 1995a). FSH-treated Sertoli cells contains immunoreactive StAR but neither protein nor mRNA of CYP11A. This is suggestive that the function of StAR protein is not providing cholesterol to CYP11A-catalysed reaction and the possible function is to enhance free fatty acid uptake into mitochondria (Gregory and DePhilip, 1998).

1.7.1.8 StAR-related protein

MLN64, a 50 kDa protein, is highly expressed in certain carcinomas and localised within bundle-like structure which distributed throughout the cytoplasm and condensed in a perinuclear patch. Its C terminus shares significant homology with StAR protein and the F26F4.4 protein of *Caenorhabditis elegans*. This novel protein domain was termed as **S**tAR **H**omology **D**omain (SHD). MLN64 expressed in human placenta and brain might play a similar role to StAR protein in promoting steroidogenesis by virtue of its homology to StAR (Moog-Lutz et al., 1997; Watari et al., 1997). Moreover, there is homology between StAR and members of the RhoGAP family of GTPase activating protein (Arakane et al., 1998).

1.7.2 Transcriptional regulation of StAR gene

The transcription factor has emerged as an essential regulator of steroidogenic cell functions in the adrenal and the gonads (Ikeda et al., 1994; Parker and Schimmer, 1996; Parker and Schimmer, 1997). StAR gene transcription is regulated by the orphan nuclear receptor, steroidogenic factor 1 (SF-1), which is also termed adrenal 4-binding protein (Ad4BP). The promoter of genes regulated by SF-1 contains a consensus, i. e. AGGTCA-derived motif (Bertherat, 1998). SF-1 is a global regulator

within the hypothalamic-pituitary-adrenal and -gonadal axes (Parker and Schimmer, 1996).

Cloning and sequencing of SF-1 cDNA established that SF-1 is an orphan member of the nuclear receptor family of ligand-inducible transcription factors (Morohashi et al., 1992). Recently, certain oxysterols have been considered as SF-1 ligands that can stimulate SF-1-dependent transcription (Lala et al., 1997). Transcriptional activation of StAR gene by a SF-1-dependent mechanism may represent a common approach for the actions of ACTH and Ang II in stimulating steroidogenesis (Clark and Combs, 1999). Moreover, SF-1 appears to be involved in cAMP regulation of transcription in the adrenal cortex (Bertherat, 1998).

Secretory protein 1 (Sp1) is a widely expressed member of the zinc finger family of transcription factors (Kadonaga et al., 1987). Several putative Sp1 binding sites have been identified in the human StAR promoter, which may participate in regulation of *StAR* gene (Sugawara et al., 1995b; Reinhart et al., 1999).

In contrast, a powerful repressor, DAX-1 (dosage-sensitive sex reversal adrenal phoplasic congenita critical region on the X-chromosome, gene-1) was shown to inhibit StAR gene expression by binding to a hairpin structure in a region of single-stranded DNA found in the promoter region of the *StAR* gene (Zazopoulos et al., 1997).

1.7.3 Translation regulation and post-translational modification of StAR protein

The activity of StAR can be increased by a co- or post-translational modification. The findings by Arakane and co-worker demonstrate that serine 194/195, a potential site of phosphorylation mediated by protein kinase A, is an important residue in StAR. Phosphorylation of StAR may be part of the mechanism of the immediate increase in steroid production following tropic hormonal stimulation (Arakane et al., 1997), a process that is essential to maximising StAR activity (Kallen et al., 1998a).

1.8 HYPOTHESIS AND GENERAL AIMS

The crucial role of StAR protein in steroidogenesis by various tissues is well recognised (Stocco, 2000; Stocco and Clark, 1997). However, the mechanisms controlling acute ACTH-induced cortisol section by adrenal ZF cells have not yet been clearly established, especially under physiologically relevant conditions which is vital in elucidating the acute response to tropic hormones.

StAR protein may regulate the onset of the elevated steroidogenesis and the maintenance of high level steroidogenesis through distinct mechanisms. The initiation of steroidogenesis may be induced by pre-existing StAR via the post-translation modification, whereas the maintenance of steroidogenesis relies on newly synthesised StAR protein produced from mRNA translation. The signalling systems mediating steroidogenesis at different levels of hormonal treatment are activated by a more complicated mechanism (e.g. various signalling pathways and/or cross-talks between intracellular signals).

This work has focused on the primary bovine adrenal ZF cells, an excellent *in vitro* model for studying steroidogenesis, which addresses not only the questions of StAR's function in hormonal-stimulated steroidogenesis but also the signalling systems involved the activation of steroid production. The general aims were:

- To observe the expression of StAR protein in the absence and the presence of various concentrations of ACTH.
- To determine the temporal relationships between StAR synthesis and steroid production in response to trophic hormone (ACTH and Ang II) treatments.
- To compare the intracellular signalling systems of steroidogenesis activated by physiological (10^{-12} M) and pharmacological (10^{-8} M) concentrations of ACTH.
- To evaluate the role of protein kinase C in ACTH-induced steroidogenesis.

CHAPTER 2 METHODS AND MATERIALS

2.1 MATERIAL SUPPLIES

2.1.1 Materials obtained from commercial sources

Chemicals, reagents and media from commercial sources were listed in Appendix I.

2.1.2 Materials from other sources

Bovine peptide StAR antigen and sheep anti-bovine peptide StAR antibody (primary antibody) were produced for Prof. Ian Mason, Department of Clinical Biochemistry, University of Edinburgh and Prof. Paul Stewart, Department of Medicine, University of Birmingham by The Binding Site (P. O. BOX 4073, Birmingham B29 6AT, UK). The StAR antigen was a 26-amino acid peptide (position 82-107 residues of StAR protein), consisting of A-M-Q-R-A-L-G-I-L-K-D-Q-E-G-W-K-K-E-S-R-Q-A-N-G-D-E (Hartung et al., 1995). The primary antibody was raised in sheep using this peptide in a proprietary matrix.

Rabbit anti-mouse recombinant antibody raised against a GST-fusion protein (StAR cDNA, bp 208-1467) was kindly supplied by Dr. Dale B. Hales, Department of Physiology & Biophysics, University of Illinois at Chicago, USA (Clark et al., 1997; LeHoux et al., 1999).

Bovine ovary and ovarian theca cells were kindly were kindly provided by Dr. Tony Bramley (Centre for Reproductive Biology, University of Edinburgh) and Prof. Richard Ivell (Institute for Hormone and Fertility Research, University of Hamburg, Hamburg, Germany) respectively.

Rabbit anti-cAMP antibody (batch R1B7) was generously provided by Dr. Brent C Williams, Department of Medicine, University of Edinburgh. [^{125}I]-cAMP tracer was iodinated and supplied by Dr. F. Antoni, Department of Neuroscience, University of Edinburgh.

2.2 CELL CULTURE MANIPULATION AND HORMONAL TREATMENT REGIMES

2.2.1 Preparation of primary bovine adrenal ZF cells

i) Protocol 1: Isolation of adrenal ZF cells using collagenase digestion

Fresh bovine adrenal glands were collected from the local abattoir in Edinburgh (January-May 1998) and the abattoir in Linlithgow, Scotland (June 1998-present). Cattle (steers and heifers) were aged from 12 to 30 months. After being trimmed of fat, the adrenal glands were kept in a beaker with sufficient sterile 0.2% (w/v) BSA/EBSS. Approximately 100 μm slices of tissue were carefully cut using a Staddle-Riggs Microtome. The first slice containing ZG cells and some ZF cells was discarded. The second and third slices representing mainly ZF cells were collected for isolation.

Tissue slices were minced with scissors in EBSS and repeatedly washed with the same buffer until most of the red blood cells were removed and then digested using 2 mg/ml collagenase in 0.2% BSA/EBSS at 37°C for 2 hr with occasionally shaking. The digestion mixture was filtered through a coarse-mesh nylon gauze (200 μm) and a medium-mesh (100 μm), then centrifuged at 400 x g for 20 min. The cell pellet was suspended in 0.2% BSA/EBSS, and the suspension was filtered by a fine-mesh gauze (30 μm). Subsequently the suspension was loaded into a Sephadex column (4 cm in height G-10 plus 0.5 cm in height G-50 on top). Cell debris, blood cells and smaller ZR cells were washed off slowly with 0.2% BSA/EBSS while the adrenal ZF cells were trapped by Sephadex G-10. Afterwards, the trapped cells and Sephadex mixture were gently resuspended with the same buffer and filtered through a fine-mesh gauze to trap the Sephadex. Finally, cells were collected by centrifuging (400 x g, 30 min, room temperature) and resuspended in the growth medium, consisting of Ham's F10 containing 10% (v/v) process serum replacement-1 and 1% antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B).

Cells were plated out at a density of 1.5 x 10^6 cells per well (35 mm in diameter, 6-well-plate) and incubated at 37°C in 5% CO_2. The day of cell isolation is described as Day 0.

ii) Protocol 2: Isolation of adrenal ZF cells using trypsin digestion

Bovine adrenal glands were sectioned as described in Protocol 1. The slices were rinsed with warm (37°C) DMEM/F12 medium containing 1% antibiotics (see protocol 1), then transferred to the siliconised glass flasks (25 ml and 50 ml, Bello Glass, Inc., NJ, USA or Wheaton Science Products, NJ, USA). The supernatant was discarded after the tissue was slowly stirred with a siliconised glass rod plus a paddle-shape accessory in 0.25% trypsin digestion solution for 15 min. This procedure was repeated once. Then tissue was slowly stirred in 20-30 ml of warm (37°C) digestion solution for about 30 min. The suspension was transferred to a beaker and the slice remains were broken down with the end of a 5 ml pipette (the end broken off). The digestion procedure was repeated once or possibly twice until only connective tissue was left. The suspension was filtered with a coarse pan filter and centrifuge at 400 x g for 20 min (room temperature). Each cell pellet was washed in 5-10 ml of the growth medium. Finally, cells were resuspended and plated out in a similar manner to collagenase-digested cells.

iii) Maintenance of primary bovine zona fasciculata cells

On the day following isolation (Day 1), cells were washed with EBSS to remove debris and unattached cells before addition of fresh growth medium. On Day 2 the medium was replaced with Ham's F10 containing 0.2% BSA and 1% antibiotics, i.e. serum-free medium.

2.2.2 Hormonal treatments

All experiments of bovine adrenal ZF cells were conducted using 6-well plate (1.5x10^6 cells) and 3 ml medium.

i) ACTH treatments on separate day of cell culture

Cells were treated with 10^{-8} M ACTH on each day respectively from Day 0 to Day 4. At 6 hr and 24 hr time points, cells were gently scraped off from dishes in EBSS or PBS, then the suspension was transferred to a 20 ml universal tube and spun at 600 x g for 10 min. The cell pellets were washed with EBSS or PBS, then stored at -70°C.

ii) Concentration-response analyses

3-Day cultured cells were treated with various concentrations of ACTH ranging from 10^{-14} M to 10^{-7} M at 10 times intervals. At 1 and 6 hr, 0.5 ml of medium was transferred to a glass tube for cAMP measurement (see details in Section 2.6.1) and the remains were collected for cortisol assay. Cells were harvested as described in section 2.2.2.i.

iii) ACTH and Ang II Time courses

3-Day cultured cells were treated with 10^{-8} M ACTH, 10^{-12} M ACTH, 10^{-8} M Ang II, the combined Ang II and ACTH (10^{-12} M) respectively. Cells and media were collected at 0.5, 1, 2, 4, 6, 8, 12 and 24 hr.

iv) Inhibition experiments

4-Bromophenacyl bromide, nordihydroguaiaretic acid and indomethacin were dissolved in absolute ethanol, then diluted to 0.05-0.1% (v/v) in the media. Bisindolylmaleimide I (GF 109203X) in dimethyl sulphoxide (DMSO), then diluted to 0.05-0.1% (v/v) in the media. After being preincubated with various compounds for 15 or 60 min, cells were treated with 10^{-12} M or 10^{-8} M ACTH in the presence of compounds. At 1 hr and 6 hr, after collecting media, cells were washed with EBSS twice. 6-well plates were overlaid initially with liquid nitrogen and stored at -70°C. This was an alternative method for harvesting cells, especially when larger number of samples were involved.

v) Phorbol ester treatment

Phorbol-12-myristate 13-acetate (PMA) was dissolved in DMSO and diluted to 0.05% (v/v) in the media. 3-Day cultured cells were treated with 10^{-8}, 10^{-7} and 10^{-6} M PMA respectively; and 10^{-8} M ACTH plus various concentrations of PMA. Cells and media were collected at 6 and 24 hr.

2.3 PREPARATION OF PROTEIN SAMPLES

2.3.1 Protein preparation from tissues and cells

i) Total cellular extract from bovine adrenal tissue

Tissues were homogenised in the lysis buffer, i.e. PBS buffer containing 1% deoxycholic acid (sodium salt) and 0.1% SDS (Naville et al., 1991). The volume of the homogenising/lysis buffer containing protease inhibitors (in the ratio of 1:25) was 5 times (v/w) of the tissue weight. Proteins were extracted by centrifuging at 800 x g for 10 min and subsequently centrifuging again at 10,000 x g for 15 min (4°C). The protein concentration was estimated (Bradford, 1976, see details in section 2.3.3). The protein samples were combined with the boiling mix in the ratio of 2:1 (in volume). The boiling mix consisted of 1% SDS, 4% glycerol, 0.3% mercaptoethanol and 0.7% saturated bromophenol blue solution. Finally, the mixture was boiled for 2-5 min. After cooling to room temperature, the samples were loaded on to gels or stored at -20°C.

ii) Preparation of mitochondria-enriched fractions from bovine adrenal tissue

Tissues were homogenised in 5 times (v/w) TSE buffer (10 mM **T**ris, 25 M **S**ucrose, and 0.1 mM **E**DTA, pH 7.4) with a glass tube and a Teflon pestle. Mitochondria-enriched fractions were extracted by centrifuging at 600 x g for 30 min (4°C), followed by centrifuging the resultant supernatant at 9000 x g for 30 min (4°C). The mitochondrial pellet was solubilised in the lysis buffer.

iii) Total cellular extract from primary BAC cells

Cells were homogenised in the lysis buffer using a Soniprep, e.g. 150-200 µl per 1.5 x 10^6 cells. Afterwards, other steps are the same as those of the total protein preparation of the bovine adrenal cortex tissue.

iv) Preparation of mitochondria-enriched fraction from primary BAC cells

Fresh cell pellets were homogenised in TSE buffer (10 mM **T**ris, 0.25 M **S**ucrose and 0.1 mM **E**DTA, pH 7.4). Mitochondrial protein was prepared using the same procedure as those of the bovine adrenal tissue.

2.3.2 Crude subcellular fractionation

i) Homogenisation

Fresh bovine adrenal tissues (ZG, ZFR and medulla) were homogenised respectively using a Teflon homogeniser in 5 times (v/w) TS buffer (5 mM **T**ris and 0.275 M **S**ucrose, pH 7.4).

ii) Nuclei extraction

The homogenate mixture was spun at 600 x g for 20 min (4°C), then supernatant was transferred to a fresh tube and the nuclear pellet was washed with TS buffer. After centrifugation, the pellet was solubilised in the lysis buffer.

iii) Mitochondrial -enriched extraction

The supernatant from the previous step was spun at 9000 x g for 30 min (4°C), then this supernatant was transferred to special centrifuge tubes. The mitochondrial pellet was washed with TS buffer and solubilised in lysis buffer.

iv) Cytosol and microsome extraction

The supernatant from the previous step (iii) was centrifuged at 100,000 x g for 1 hr (4°C) using an ultracentrifuge. This supernatant was considered a cytosol fraction and the pellet was a microsomal fraction. Solubilisation of microsomes was conducted as for the mitochondrial fraction.

2.3.3 Protein measurement by the Bradford assay

i) Working principle

The Bradford assay (Bradford, 1976) is based on the observation that Coomassie Brilliant Blue G-250 exists in two different colour forms, red and blue. The red form is converted to the blue form upon the binding of the dye to protein (Reisner et al., 1975), which causes a shift in the absorption maximum of the dye from 465 to 595 nm. The signal can therefore be monitored as the protein-dye complex has a high extinction coefficient thus leading to great sensitivity.

ii) Standards

Stock BSA (1000 mg/L in PBS buffer) was diluted in a 1:10 ratio with distilled water to obtain 2ml of 100 mg/L standard, then this standard was diluted further to 20, 40, 60 and 80 mg/L. The protein dilution included 0, 20, 40, 60, 80 and 100 mg/L for generating a standard curve.

iii) Bradford assay

The samples were diluted in an appropriate manner (for example 1:50 for cell homogenate) with distilled water and vortexed before the assay. The standards were used as the calibration curve to determine the unknown protein concentration. The assay was carried out by using a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). Samples greater than 100 mg/L were diluted in series with distilled water until they fell into the range of the standard curve (the minimum level was not below 10 mg/L).

2.4 WESTERN IMMUNOBLOTTING

2.4.1 SDS-PAGE and protein transfer to membranes

SDS-PAGE is based on the method by Laemmli (Laemmli, 1970). One-dimensional electrophoresis was performed in a 12.5% SDS gel (Table 2-4-1).

Table 2-4-1 Components of the SDS-PAGE gel

	Resolving gel	
Solution components volume in ml	2 x Large gel (12.5%)	2 x mini-gel (12.5%)
Distilled water	17.00	3.40
1.0 M Tris/HCl, pH 8.85	30.00	6.00
30% Acrylamide plus 0.8% Bis-acrylamide	32.00	6.40
10% SDS	0.80	0.16
TEMED	0.15	0.03
10% AMPS (daily fresh)	0.15	0.03
	Stacking gel	
Distilled water	32.00	6.40
0.375 M Tris/HCl, pH 6.8	20.00	4.00
30% Acrylamide plus 0.8% Bis-acrylamide	7.20	1.44
10% SDS	0.60	0.12
TEMED	0.20	0.04
10% AMPS (daily fresh)	0.20	0.04
Note: The size of molecular mass marker vary among various batch products as the controls for the measurement are different.		

The proteins were electrophoretically transferred on to Immobilon-P polyvinylidene difluoride (PVDF) membrane which had been pre-wetted in methanol then equilibrated in transfer buffer (25 mM Tris and 192 mM glycine). The transfer conditions for 12.5% mini-gels were: 250 mA (constant) and 1.5-2 hr running time. For 12.5% large gels, the conditions were 45-50 voltage (constant) and 4 hr. Afterwards, the membranes were placed in PBS buffer containing 5-10% non-fat milk or Pierce blocking buffer overnight at 4°C.

2.4.2 Enhanced chemiluminescence (ECL) detection

i) Working principle

ECL is a light emitting non-radioactive method for detection of immobilised specific antigens (proteins of interest) which bond directly or indirectly with horseradish peroxidase-labelled (or biotinylated) antibody (Fig. 2-4-2).

ECL is achieved by performing the oxidation of luminol by the horseradish peroxidase in the presence of chemical enhancers. This has the effect of increasing the light output approximately 1000 fold and extending the time of light emission (ECL protocols provided by Amersham Life Science).

ii) Primary antibody incubation

After blocking overnight, the membrane was washed with PBS buffer containing 0.05% (v/v) Tween 20 on an orbital shaker at room temperature. The routine washing procedure for ECL detection was of 5, 10, 15 and 15 min with fresh buffer on each occasion. Subsequently, the membrane was incubated with the primary antibody for 1 hr. The antibody was diluted in PBS or PBS/10% Pierce blocking buffer containing 0.5-5% non-fat milk. The optimal dilution (e.g. 1:10,000 for StAR peptide sheep antibody) and buffer composition were determined by testing various combined conditions.

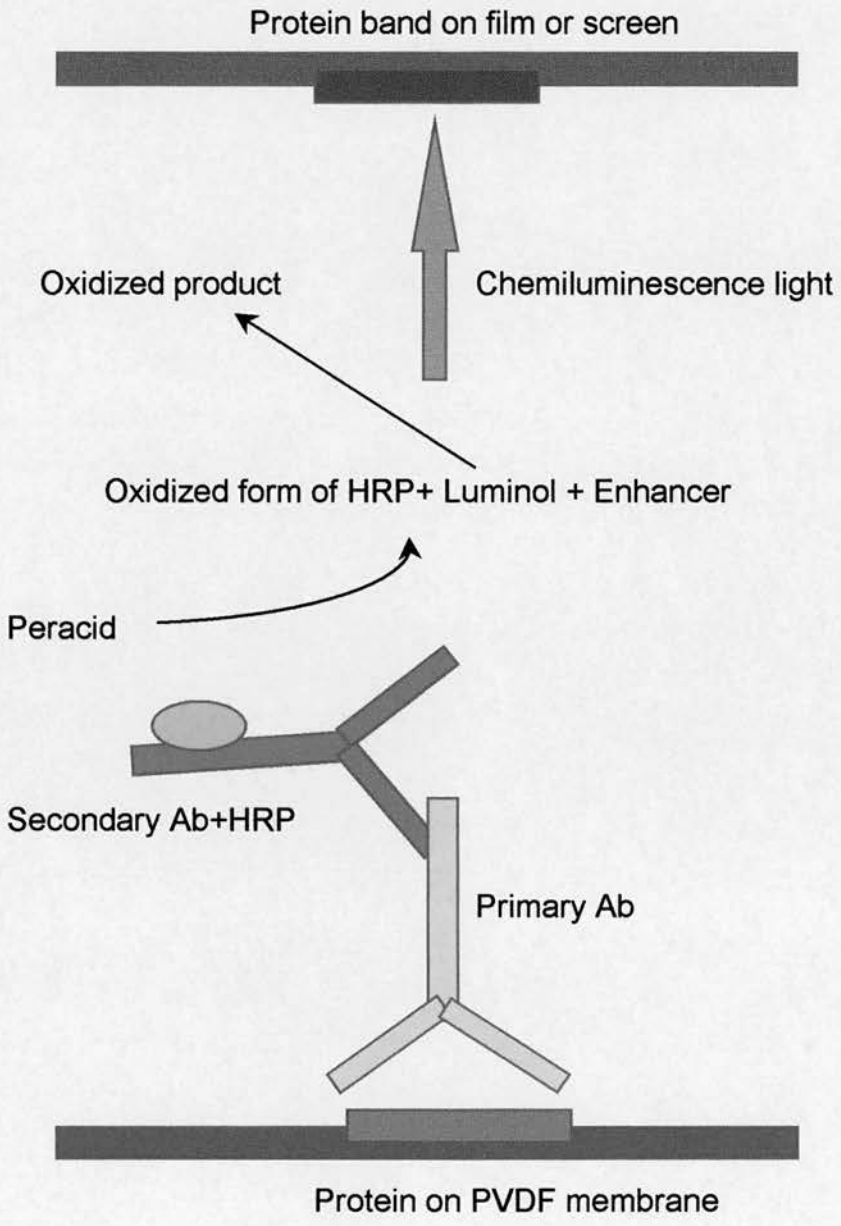

Fig. 2-4-2 Schematic representation of the principle of enhanced chemiluminescence (ECL) Western immunoblot Ab: Antibody; HRP: Horseradish peroxidase. Adapted from an Amersham ECL schematic.

iii) Secondary antibody incubation

The membrane was washed with PBS buffer according to the routine washing procedure. The excess primary antibody was removed completely. The secondary antibody conjugated to horseradish peroxidase (HRP) was diluted and applied to the membranes using the same procedures as those of the primary antibody (e.g. 1:100,000 for donkey anti-sheep/goat antibody). After 1 hr incubation, the membrane was washed following the routinely procedure except that Tween 20 was omitted from the last wash step.

iv) ECL Detection

ECL detection was carried out by following a protocol provided by Pierce Ltd: SuperSignal West Dura Stable Peroxide Solution was equally mixed with West Dura Luminol/Enhancer solution to sufficiently cover the membranes. The membranes were incubated for 5 min at room temperature. After draining off excess detection reagent, the membranes (protein side up) were placed on a piece of SaranWrap and sealed with autoclave tape. The signal was firstly visualised by exposing the membranes to X-OMAT LS film or X-OMAT film (more sensitive) for 1 min or longer/shorter depending on the working conditions such as milk concentration and dilution of antibodies. Afterwards, the wrapped membrane was exposed to a chemiluminescence screen (Bio-Rad Image screen-CH) for 3-4 hr. In the first instance, the screen (20 x 25 cm) was scanned (Bio-Rad, Model GS-525) at a resolution of 800 μm. Regions of interest were marked manually and scanned at a resolution of 200 or 100 μm to obtain a close up view of the image picture.

For the Amersham ECL kit, equal volumes of solution 1 and solution 2 were mixed before use. The protein side of the membrane was incubated with the detection reagent for 1 min at room temperature. Other procedures were the same as those of Pierce ECL detection.

2.5 CORTISOL MEASUREMENT BY RADIOIMMUNOASSAY ('IN HOUSE' ASSAY)

2.5.1 Working Principle

Radioimmunoassay (RIA) is a competitive immunoassay in which the antigen labelled with a radioisotope competes with the unlabelled antigen (known standards or unknown samples) to bind to a specific antibody. After an appropriate reaction time, antibody-bound and unbound labelled antigens are separated by double antibody precipitation and centrifugation, or other techniques (Yalow, 1992).

2.5.2 Radioimmunoassay for cortisol

i) Preparation of preprecipitate (PPT)

Firstly, a 1 ml vial of lyophilised sheep anti-cortisol primary antibody was reconstituted with distilled water for 1 hr. Afterwards, the following components were mixed in a universal tube and left in the refrigerator overnight: 650 µl sheep anti-cortisol, 530 µl normal sheep serum, 15 ml Donkey anti-sheep/goat IgG secondary antibody and 10 ml 0.1 M citrate buffer. On the following day, the mixture was centrifuged at 230 x g for 15 min (4°C), then the pellet resuspended in 500 ml of citrate buffer and stored in refrigerator.

ii) Standard cortisol

Cortisol was dissolved in absolute ethanol to 10 mM and stored in -70°C. The stock solution was diluted with Ham's F10 medium containing 0.2% BSA to obtain the following standards: 0, 1, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 nM; and 8, 80 and 800 nM as controls (concentration known samples).

iii) Working tracer solution

The stock tracer (10 μCi, 500 μl of methanol was added to 100 μl of cortisol-3-com-(2-[^{125}I] iodohistamine) was diluted with 0.1 M citrate buffer in the ratio of 1: 2800.

iv) Cortisol measurement

All reagents, standards and samples were warmed to room temperature. Samples were centrifuged briefly to remove any debris if it was necessary and the supernatant was retained for assay. Certain samples were required to be diluted further before the RIA.

Each 100 μl o f standard or sample was added to 700 μl working solution of [^{125}I]-cortisol tracer in duplicate using an automatical diluter (HAMILTON, Switzerland), then added 250 μl of PPT. The assay tubes were shaken on a multivortex an d incubated at 37°C for 1.5-2.0 hr. After spinning for 30 min at 1200 x g (4°C) the supernatant was discarded, then the tubes were blotted on wadding. The retained [^{125}I]-cortisol was counted for 2 min by Gamma Counter (LKB 1261 Multigamma, Pharmacia, Turku, Finland) and cortisol contents were determined against a standard curve.

The intra-assay of coefficient of variation (%CV) was less than 10%. The inter-assay %CV was less than 10% for the range from 50 nM to 2000 nM and 15% for the range from 10 to 50 nM (Vanessa J. Cobb, 1999, PhD thesis. Department of Clinical Biochemistry, University of Edinburgh).

2.6 cAMP MEASUREMENT BY RADIOIMMUNOASSAY ('IN HOUSE' ASSAY)

2.6.1 Acetylation of samples

To increase the accuracy of measurement of low levels of cAMP, the medium samples were required to be acetylated within 1 hour after collection. 500 μl of medium was transferred to a glass tube and acidified to pH 5.0 by the addition of 5 μl of 20% (v/v) acetic acid prior to acetylation. Subsequently, 15 μl of the acetylation

reagent (triethylamine and acetic anhydride in a ratio of 2:1 (v/v) was added to each sample. All samples then were stored at -20°C prior to assay.

2.6.2 Radioimmunoassay for cAMP

i) Primary antibody

Rabbit anti-cAMP antibody (batch R1B7) was diluted 1:100 (v/v) in 50 mM sodium acetate buffer, pH 5.0 (add 0.1% BSA prior to assay).

ii) Secondary antibody

20 ml of Donkey anti-rabbit serum was mixed with 1.5 ml normal rabbit serum in a universal tube and left in the refrigerator overnight. On the next day the mixture was spun at 230 x g for 15 min and resuspended in 80 ml of 0.05 M phosphate buffer.

iii) Standards

Stock cAMP (10 mM in acetate buffer without BSA) was diluted with Ham's F10 medium containing 0.2% BSA to give 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 nM samples for a standard curve and 320 nM for blank (non-specific binding) plus 0.04, 0.08,0.16 and 1.6 nM for control. All standards were acetylated in glass tubes prior to assay.

iv) cAMP measurement

50 µl of medium samples and standards were added in duplicate with 100 µl of ^{125}I-cAMP tracer (in acetate buffer containing 0.1% BSA, approximately 5000cpm), then 100 µl primary antibody (1:100 in the same buffer as the tracer). This mixture was incubated overnight at 4ºC. On the next day, after the addition of 100 µl secondary antibody, the tubes were vigorously shaken for 1 hr. Then, 1.5 ml of wash solution (a small amount of microcrystalline cellulose plus 1.5 ml of BriJ detergent in 1 litre of distilled water) was added to the tubes and centrifuged at 1200 x g for 30 min (4°C). After discarding supernatant the tubes were blotted on wadding. The [^{125}I]-

cAMP signal was counted for 6 min in the Gamma Counter and cAMP contents were determined in duplicate against the standard curve.

The minimum detectable level was 0.33 nM and the intra-assay %CV was less than 10% over the working range of the assay.

2.7 DATA ANALYSIS

Unless otherwise stated, data presented in Chapter 4 were obtained from 3-4 independent experiments; data presented in Chapter 5 were obtained from an experiment conducted in triplicate and the same experiment was repeated twice and data in Chapter 6 (except PMA treatments) were obtained from an experiment conducted in triplicate. Values were expressed as mean ± SEM. The mean values were compared by analysis of variance (ANOVA), followed by Student-Newman-Keuls (SNK) test. Non-normal distribution data were transformed by log 10 or square root. A value of $P<0.05$ was considered as statistically significant, $P<0.01$ and $P<0.001$ were considered as highly significant. The analysis was performed by using Minitab Version 12 (Minitab Inc., PA, USA) and GraphPad InStat Version 3.0 (GraphPad Spftware Inc., San Diego, CA, USA).

CHAPTER 3 CHARACTERISATION OF A POLYCLONAL ANTIBODY GENERATED AGAINST A PEPTIDE SEQUENCE OF BOVINE STEROIDOGENIC ACUTE REGULATORY (StAR) PROTEIN

3.1 INTRODUCTION

StAR protein plays a crucial role in the regulation of steroidogenesis, facilitating the translocation of cholesterol from the outer to the inner mitochondrial membrane where CYP11A, the first enzyme of the steroid synthetic pathway, is located. Western blot analysis using ECL or autoradiography is a basic method for visualising proteins, which is extremely useful for the identification and the quantitation of target protein in complex mixture of proteins. Reagents for detecting StAR protein, reported to date, are summarised below:

Three distinct polyclonal primary antibodies directed against StAR protein have been described in the literature. A rabbit anti-mouse peptide StAR antibody produced by Dr. DM Stocco (Texas Tech University Health Science Center, Lubbork, USA) has been used in a number of laboratories. This is an anti-peptide antibody generated in rabbit against a peptide sequence (amino acid 88-98) of mouse StAR protein (Clark et al, 1994; Pescador et al, 1996; Cherradi et al, 1997; Ronen-Fuhrmann et al, 1998; Manna et al, 1998; LeHoux et al, 1998; Roy et al, 2000). The second one is a rabbit anti-human recombinant StAR antibody which was obtained with a StAR protein from a cDNA encoding amino acid residues 63-285. The localisation of StAR protein in human adult and fetal tissues was established (Pollack et al, 1997) and the several forms of StAR protein under different physicochemical conditions (e.g. pH and urea concentrations) were observed using this anti-recombinant StAR antibody (Bose et al, 1999). In addition, a rabbit anti-mouse recombinant StAR antibody was generated using a StAR-GST (*Schistosoma japonicum* glutathione S-transferase) fusion protein from mouse StAR cDNA fragment (position 208-1467 bp). This primary antibody was used for Western immunoblotting after SDS-PAGE and 2-D gel electrophoresis (LeHoux et al, 1999).

Secondary antibodies conjugated with horseradish peroxidase (HRP) have been used to visualise StAR protein-antibody complexes; donkey anti-rabbit IgG conjugated with HRP (Clark et al, 1994; Pescador et al, 1996; Roy et al, 2000) and goat anti-rabbit IgG conjugated with HRP (Bose et al, 1998; Ariyoshi et al, 1998). In addition, radioisotopically-labelled secondary antibody such as [^{125}I]-goat anti-rabbit IgG was also used for immunocytochemical and immunoblot analysis of StAR protein (Nishikawa et al, 1996; Nishikawa et al, 1997).

Several ECL detection kits are available for Western immunoblotting. The Amersham ECL kit has been extensively used for the studies on StAR protein in MA-10 Leydig tumour cells (Manna et al, 1998), the rat adrenal (LeHoux et al, 1999) and the bovine adrenal ZFR (Roy et al, 2000). The Pierce SuperSignal, a relatively new ECL kit, was employed to estimate StAR protein in rat ovarian luteal cells (Sandhoff et al, 1998).

Because the primary antibodies described above were available in insufficient amounts to complete this and other projects and no primary antibody against StAR protein is yet available from a commercial source, it was necessary to generate a sufficient supply of antibody for use in our laboratory. The sheep anti-bovine peptide antibody, a novel antibody generated against bovine StAR protein amino acids 82-107 has been explored in some detail. This strategy necessitated the characterisation and development of methodology to establish optimisation of detection conditions for this antibody. Thus, the purposes of this work described in this chapter were:

- To optimise the experimental conditions (e.g. antibody dilution and detection reagent) to obtain a strong signal with clean background for quantification of immunoactive StAR protein band.
- To examine the antibody specificity using antigen competition test (validation test).
- To determine the subcellular distribution of StAR protein in bovine adrenal.
- To evaluate StAR protein in various bovine tissues.

3.2 OPTIMISATION OF EXPERIMENTAL CONDITIONS FOR WESTERN IMMUNOBLOT

3.2.1 Comparison of various Western immunoblot detection systems

i) Comparison between colour detection and Amersham-ECL (A-ECL) methods

Protein samples were prepared from tissues or cells and resolved on a 12.5% SDS-PAGE gel. Subsequently, proteins were electrophoretically transferred from the polyacrylamide gels on to polyvinylidene difluoride (PVDF) membranes.

StAR protein was initially detected using a colour development reagent (4-chloro-1-naphthol in methanol mixed with H_2O_2 in Tris-buffered saline, pH 7.4). This detection method, however, required a relatively high concentration of antibodies (1,000-fold dilution for the primary antibody and 2,000-fold dilution for the secondary antibody). A principal shortcoming was that the colour reaction did not provide a permanent record. The peroxidase-stained bands on the membrane faded quickly and the sensitivity of this detection was very low. Compared to colour detection, A-ECL detection was quickly revealed to be more efficient in terms of the increased antibody dilution, signal visibility and maintenance (Fig. 3-2-1a).

ii) Comparison between A-ECL and Pierce-ECL (P-ECL) detection systems

The chemiluminescence signal developed by A-ECL detection reagent was easily visualised on X-OMAT film; however, it was too faint for quantification using the Bio-Rad image analysis. As the wavelength of the light emission produced by a P-ECL kit well matches with that of the chemiluminescence screen, the P-ECL detection was therefore explored to establish a more robust approach for quantification. The results obtained with a P-ECL kit demonstrated some major advantages compared to the A-ECL kit (Fig. 3-2-1a & b): The signal produced by the P-ECL reagent was efficiently captured on the chemiluminescence screen and analysed by the Bio-Rad image system, thereby making it possible to quantify protein bands after Western blotting. The chemiluminescence signal is highly sensitive to both X-OMAT film and

the Bio-Rad image system and is of long duration (lasts about 20 hr). Furthermore, highly diluted amounts of both primary and secondary antibodies still generated a strong signal with a clean background, e.g. a 1:100,000 dilution of the secondary antibody remains effective (see details in Section 3.2.4).

3.2.2 Blocking buffer

The most appropriate blocking buffer for ECL Western immunoblot is dependent on the detection system. Western blotting using PBS buffer containing 5% non-fat milk presented strong protein bands with little background on the film (Fig. 3-2-1a). Western blotting using the Pierce blocking buffer resulted in sharp and intensive signals with a clear background (Fig. 3-2-1b). Both milk/PBS and Pierce blocking buffers were suitable for the HRP-StAR protein detecting system. However, for some detection systems such as secondary antibody conjugated with biotin, the milk blocking buffer should be avoided because milk contains variable amounts of biotin and other components which may cause non-specific binding.

3.2.3 Buffer for antibody dilution

Basically, two buffer systems were found to be useful for StAR protein Western immunoblot, i.e. PBS buffer containing milk (Fig. 3-2-3a) and PBS/10% Pierce blocking buffer containing milk (Fig. 3-2-3b). The milk concentration was varied between 1% and 5% depending on specific requirements (Fig. 3-2-3c).

3.2.4 Antibody dilution optimisation

The initial work was conducted by using the Amersham ECL detection system. However, as this reagent was not ideally suited for analysis on the Bio-Rad Image Analyser, the working conditions were modified for P-ECL detection. Sheep anti-bovine peptide antibody and donkey anti-sheep/goat IgG were diluted in various ratios to determine the best suitable conditions.

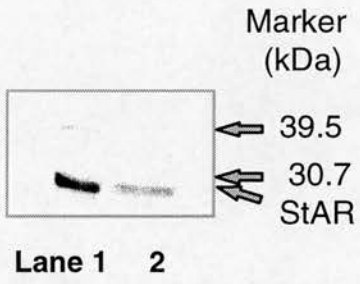

Fig. 3-2-1a StAR protein Western immunoblot using the Amersham-ECL detection system. Lane 1: Total cellular extract of freshly isolated bovine adrenal ZF cells (due to edge effect, the protein band appeared intensive); lane 2: Mitochondrial-enriched fraction of freshly isolated bovine adrenal ZF cells. The samples (25 μg of protein) were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in 5% milk/PBS buffer overnight. Sheep anti-bovine peptide antibody (1:5,000) and donkey anti-sheep/goat antibody conjugated with HRP (1: 75,000) were diluted in PBS buffer containing 5% milk, then incubated with PVDF membrane for 1 hr respectively. The membrane was washed with PBS buffer for 45 min before and after applying antibodies, and protein bands were finally visualised using a Amersham-ECL kit.

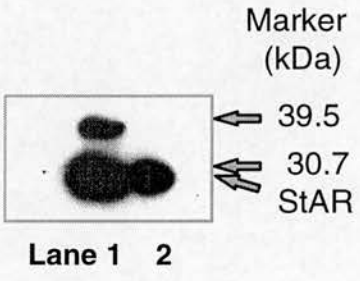

Fig. 3-2-1b StAR protein Western immnuoblot using the Pierce-ECL detection system. Lane 1: Total cellular extract of freshly isolated bovine adrenal ZF cells; lane 2: Mitochondrial-enriched fraction of freshly isolated bovine adrenal ZF cells. The samples (25 μg of protein) were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in 5% milk/PBS buffer overnight. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS buffer containing 5% milk, then incubated with PVDF membrane for 1 hr respectively. The membrane was washed with PBS buffer for 45 min before and after applying antibodies, and protein bands were finally visualised using a Pierce-ECL kit.

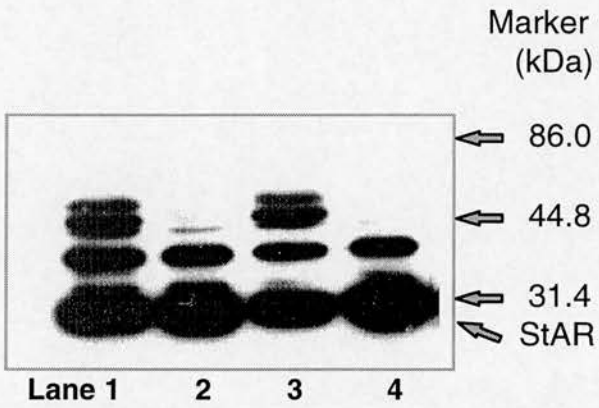

Fig. 3-2-3a StAR protein Western immunoblot using 5% milk/PBS buffer for blocking. Lane 1 and 3: Bovine adrenal cortex tissue; lane 2 and 4: Freshly isolated bovine adrenal ZF cells. All samples (25 μg of total cellular protein) were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in PBS buffer containing 5% milk overnight. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS buffer containing 3% milk, then incubated with the membrane for 1 hr respectively. Protein bands were finally visualised using a Pierce-ECL kit.

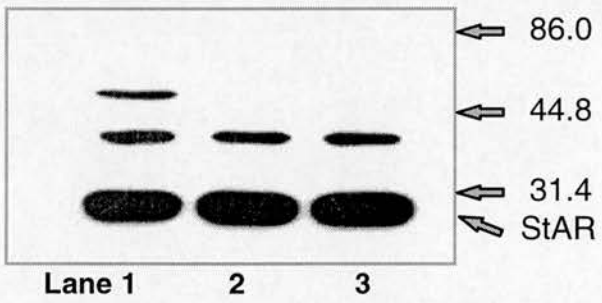

Fig. 3-2-3b StAR protein Western immunoblot using the Pierce blocking buffer for blocking. Lane 1: Bovine adrenal cortex tissue; lane 2 and 3: Freshly isolated bovine adrenal ZF cells. All samples (25 μg of total cellular protein) were resolved on a 12.5% mini-gel and blotted on to a PVDF membrane. The membrane was blocked in Pierce blocking buffer overnight. Sheep anti-bovine peptide antibody (1: 10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS/10% Pierce blocking buffer containing 3% milk, then incubated with the membrane for 1 hr respectively. Protein bands were finally visualised using a Pierce-ECL kit.

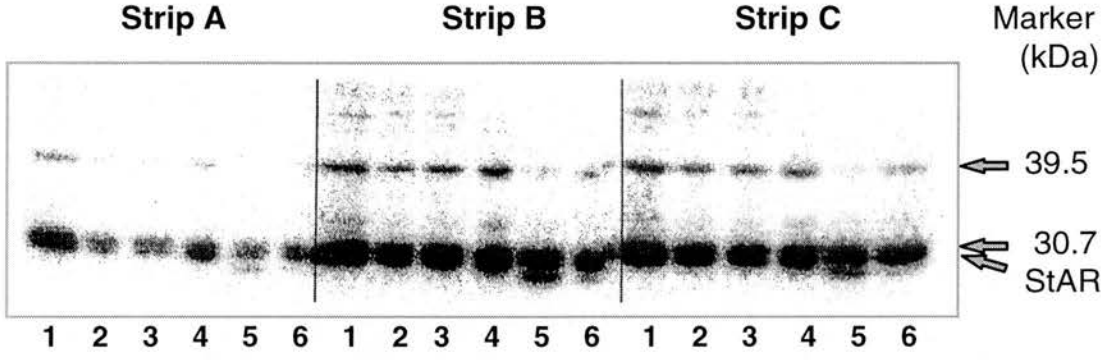

Fig. 3-2-3c A representative of Western immunoblot of comparison of milk concentration for antibody dilution (Bio-Rad image print). Strip A: PBS buffer containing 5% milk; Strip B: PBS buffer containing 3% milk; Strip C: PBS buffer containing 1% milk. Lane 1 and 3: Bovine adrenal cortex tissue; lane 2 and 6: Frozen supernatant of bovine adrenal cortex tissue; lane 4: Freshly isolated bovine adrenal ZF cells; lane 5: Frozen supernatant of adrenal ZF cells (possibly sample was partially degraded). All samples (25 μg of protein) were resolved on a 12.5% large gel and blotted on to a PVDF membrane. The membrane was blocked in Pierce blocking buffer overnight and was divided into three strips. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS buffer containing various concentrations of nonfat milk. All strips were combined together prior to the visualisation using a Pierce-ECL kit.

Fig. 3-2-4 shows a representative experiment of the optimisation, indicating that a ratio of 1:10,000 for primary antibody and 1:25,000-100,000 for secondary antibody are suitable for detecting StAR protein.

In summary, optimal conditions were very closely associated with the particular detection system used, i.e. detection reagent, blocking buffer and antibody dilution buffer.

3.2.5 Homogenising buffer

Specific homogenising buffers were chosen based on the nature of the protein fractions. The TSE buffer (10 mM **T**ris, 0.25 M **S**ucrose, and 0.1 mM **E**DTA, pH 7.4) was suitable for the homogenisation of mitochondrial-enriched fraction differentiated by centrifugation; while a PBS buffer containing 1% deoxycholic acid (sodium salt) and 0.1% SDS produced a satisfying resolution for total cellular extract preparation as 0.1% SDS dissolves all membrane proteins (Fig. 3-2-1b). The TS buffer (5 mM **T**ris-HCl, 0.275 M **S**ucrose, pH 7.4) was suitable for crude multiple fractionation (See details in Section 3.5).

3.2.6 Alternative protocols for Western immunoblot

Because the combined buffer for antibody dilution (10% Pierce blocking containing specific amounts of milk) was formulated in our laboratory, several protocols were evaluated for observing StAR protein (Table 3-2-6). Protocol A was best suited for the Bio-Rad image analysis (Fig. 3-2-6-A); however, Protocol B provided satisfying profiles for both X-OMAT film and the Bio-Rad image analysis (Fig. 3-2-6-B); while Protocol C was suitable for detecting low concentration protein and weak signals (Fig. 3-2-6-C). The range of the various dilutions necessary in these protocols is presented in Table 3-2-6.

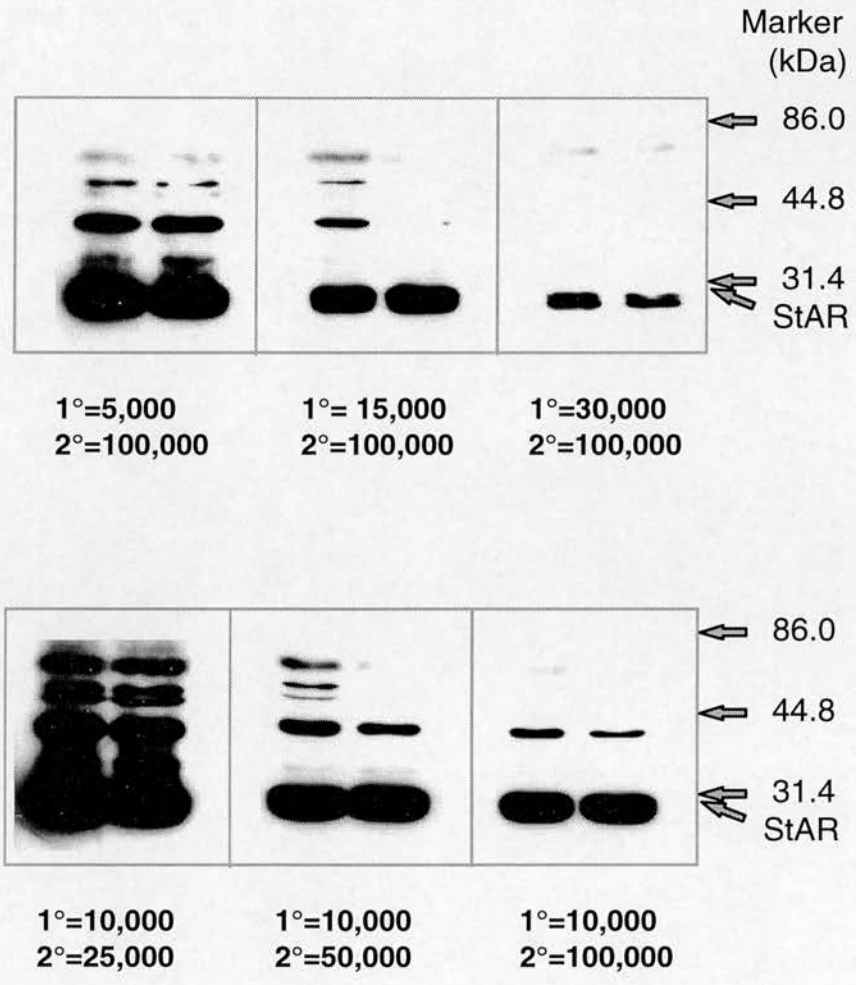

Fig. 3-2-4 StAR protein Western immunoblots-comparisons of various antibody dilution. 1°: Dilution fold of sheep anti-bovine peptide antibody; 2°: Dilution fold of donkey anti-sheep/goat antibody. All samples (25 μg of total cellular protein from bovine adrenal cortex tissue) were resolved on two 12.5% mini SDS-PAGE gels and blotted on two PVDF membranes. The membranes were blocked in Pierce blocking buffer overnight, then divided into 6 strips. All strips were incubated with the antibodies diluted with various folds in PBS/10% Pierce blocking buffer containing 3% milk. Protein bands were finally visualised using a Pierce-ECL kit.

Table 3-2-6 Alternative protocols for StAR peptide antibody and donkey anti-sheep/goat HRP antibody

Protocol	A	B	C
Dilution of anti-peptide antibody	10,000	10,000	7,500
Dilution of donkey anti-goat/sheep HRP antibody	100,000	25,000	10,000
Non-fat milk concentration (%)	0.5	2.0-3.0	1.0

3.3 QUANTIFICATION OF WESTERN IMMUNOBLOT

3.3.1 Quantification of StAR protein Western immunoblot

Fig. 3-3-1a illustrates a StAR protein (lower bands) Western immunoblot captured on the Bio-Rad CH screen. Fig. 3-3-1b was plotted as volume counts (integrated intensity) of the imaging signal versus various amounts of protein loaded on the gel. The data demonstrate that the signal intensity linearly increases with the increment of total protein amounts over a 5.0-40.0 μg range. The positive correlation ($r=0.99$, $P<0.001$) between the volume counts of StAR protein and total protein concentrations permits the use of this method for quantification of StAR protein.

3.3.2 Quantification of actin levels on Western immunoblot

To confirm the ability of quantification strategy described above for Western blotting, the levels of actin (a 42 kDa protein expressed ubiquitously in cells) were evaluated using the same method (Fig. 3-3-2a). Similar to StAR protein, there was also a positive linear correlation ($r=0.99$, $P<0.001$) between the volume counts and protein concentrations (Fig. 3-3-2b), indicating that this method fulfils the requirements of reproducibility, sensitivity and linearity.

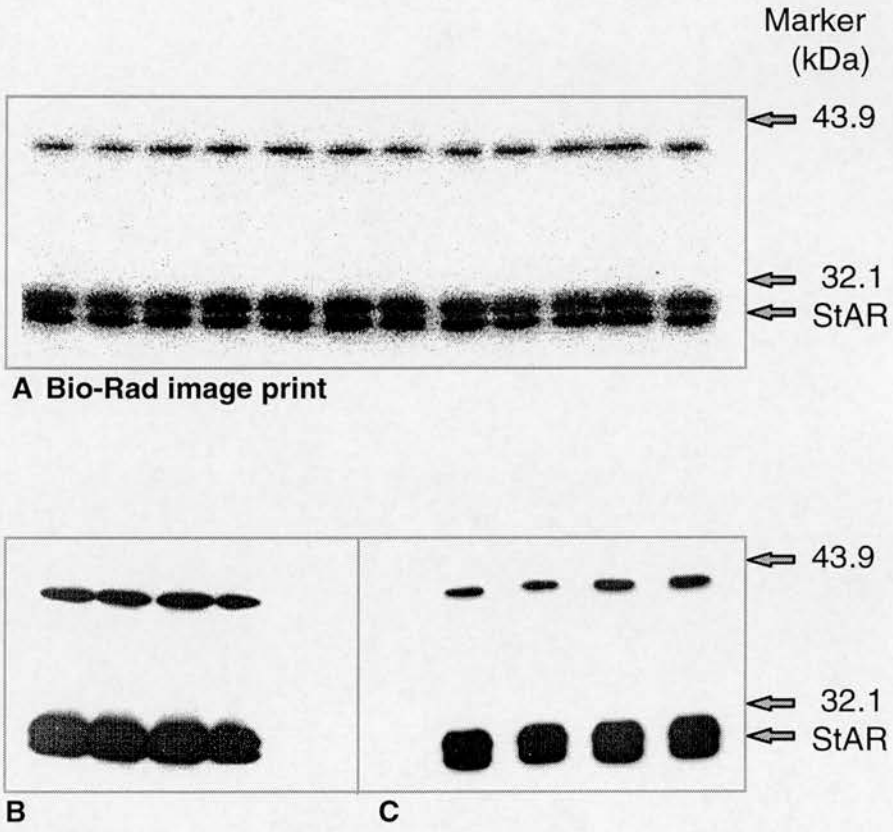

Fig. 3-2-6 StAR protein Western immunoblots using the protocol A, B and C. All samples (25 μg for A and B; 10 μg for C) are total cellular protein from freshly isolated bovine adrenal ZF cells. They were resolved on 12.5% large gels and blotted on to PVDF membranes. The membrane was blocked in Pierce blocking buffer overnight. Sheep anti-bovine peptide antibody and donkey anti-sheep/goat antibody conjugated with HRP (see details in Table 3-2-6) were diluted in PBS/10% Pierce blocking buffer containing various concentrations of milk (Table 3-2-6), then incubated with PVDF membranes for 1 hr respectively. Protein bands were finally visualised using a Pierce-ECL kit.

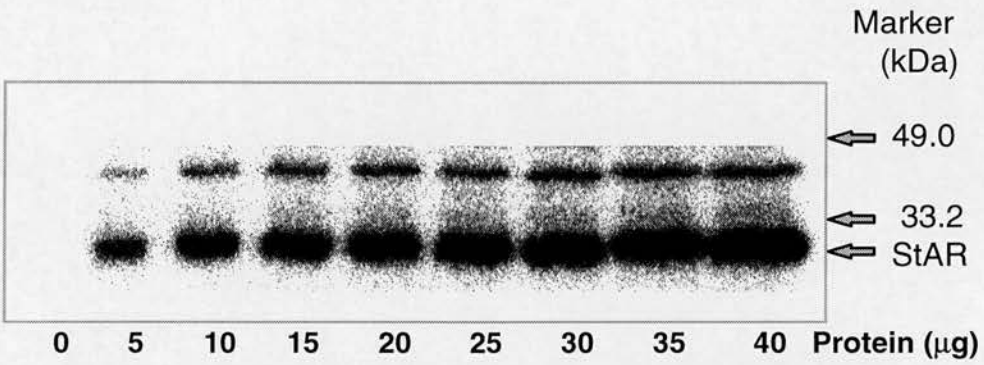

Fig. 3-3-1a Western immunoblot of StAR protein in bovine adrenal ZF cells (Bio-Rad image print). All samples are freshly isolated bovine adrenal ZF cells. Total cellular proteins ranging from 5 to 40 μg were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in Pierce blocking buffer. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk, then incubated with PVDF membrane for 1 hr respectively. The membrane was washed with PBS for 45 min before and after applying antibodies, and protein bands were finally visualised using a Pierce-ECL kit.

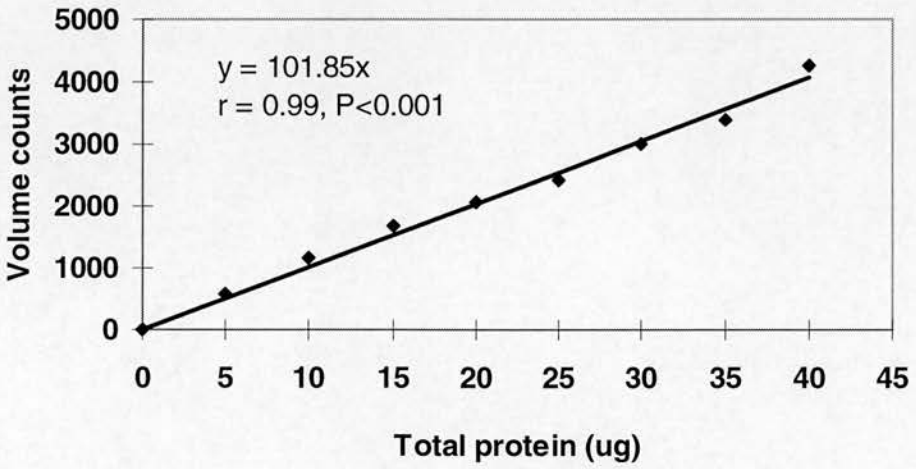

Fig. 3-3-1b Dilution curve for StAR protein of bovine adrenal ZF cells.

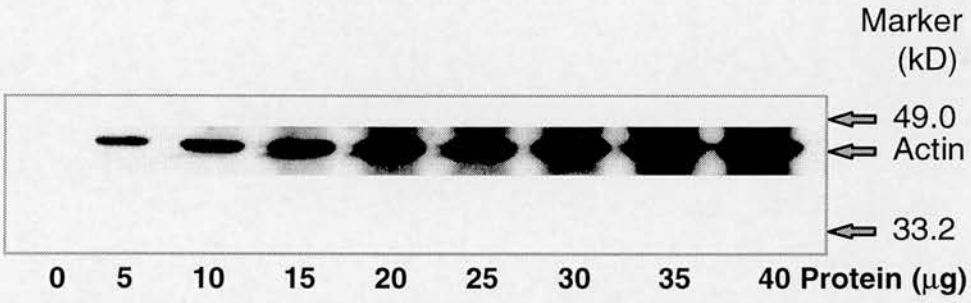

Fig. 3-3-2a Western immunoblot of actin in bovine adrenal ZF cells. All samples are freshly isolated bovine adrenal ZF cells. Total proteins ranging from 5 to 40 μg were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in Pierce blocking buffer overnight. Goat anti-human peptide actin antibody (1:2,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:20,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk, then incubated with PVDF membrane for 1 hr respectively. The membrane was washed with PBS for 45 min before and after applying antibodies, and protein bands were finally visualised using a Pierce-ECL kit.

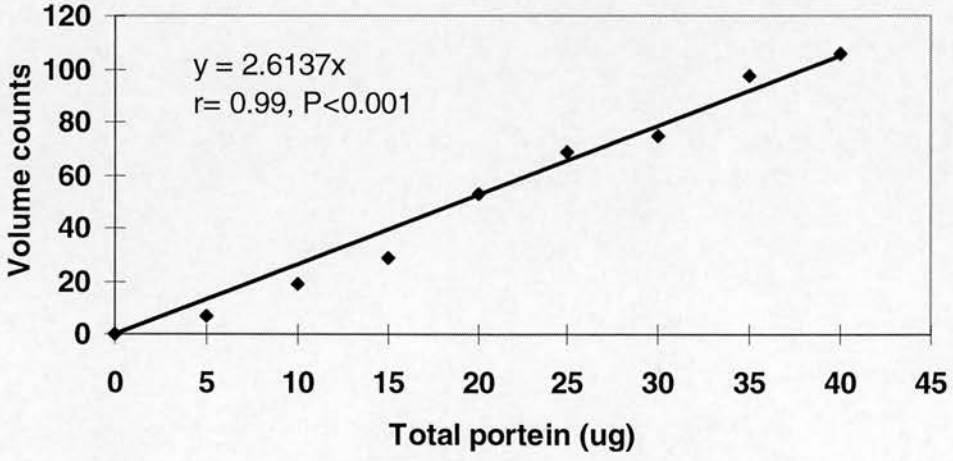

Fig. 3-3-2b Dilution curve for actin of bovine adrenal ZF cells.

3.4 VALIDATION OF ANTI-PEPTIDE StAR ANTIBODY SPECIFICITY BY ANTIGEN COMPETITION TEST

In order to determine the antibody specificity (sheep antibody versus bovine StAR protein), the effects of bovine antigen (26-amino acid peptide, see details in Section 2.1.2) on immunoblot signals were investigated by adding various amounts of antigen to the primary antibody reaction buffer. Firstly (Group A), the peptide antigen was diluted with PBS buffer at 100 times intervals (two orders of magnitude) between 10^{-17} and 10^{-5} mg/L and two PVDF membranes were divided into eight strips. Each one was incubated with the antibody reaction buffer containing a separate concentration of antigen (one control strip without the antigen). The observed protein bands (28-30 kDa and 37 kDa) were eliminated by addition of 10^{-5} mg/L antigen, while they appeared again when the antigen concentration was 10^{-7} mg/L and below (Fig. 3-4A). In a second experiment (Group B), the antigen concentration was adjusted variously between 10^{-7} and 10^{-5} mg/L. The profound difference was apparent between strips with 3×10^{-6} and 1×10^{-6} mg/L antigen, i.e. 3×10^{-6} mg/L antigen and beyond this amount resulted in no immunoreactive protein bands. StAR protein bands presented when the antigen was equal to and below 1×10^{-6} mg/L (Fig. 3-4B). Therefore, for the third test (Group C), the dilution of antigen was confined to a narrow range, which was between 1×10^{-6} and 3×10^{-6} mg/L. StAR protein bands were abolished by 2.0×10^{-6} mg/L or higher concentrations of antigen and were not affected by 1.5×10^{-6} mg/L or lower concentrations of antigen (Fig. 3-4C). These neutralisation results are strongly suggestive that the sheep antibody is specific to bovine StAR protein and recognises 28-30 kDa doublet StAR protein and possibly a StAR-associated minor band.

3.5 SUBCELLULAR FRACTIONATION OF StAR PROTEIN

3.5.1 StAR protein in bovine adrenal cortex

Subcellular fractions including nuclei, mitochondria, microsomes and cytosol were isolated from bovine adrenal ZG, ZFR and medulla respectively. The total homogenate of bovine liver was used as negative control. The results showed that

28-30 kDa protein bands existed in all the above adrenal cortex fractions, and these bands were very intense in mitochondrial-enriched fractions from ZG and ZFR (Fig. 3-5-1). As the nuclei were isolated by crude fractionation, the protein bands in this fraction may come from unbroken cells or other organelles. Apart from 28-30 kDa protein bands, two other bands (approx. 37 and 50 kDa) also existed in cytosol fraction, although the intensities of 28-30 kDa band were much decreased in the cytosol compared to mitochondrial-enriched fractions.

3.5.2 StAR protein in bovine adrenal medulla

The bovine medulla fractions were analysed in parallel with bovine adrenal cortex fractions. Unexpectedly, doublet bands (28-30 kDa) existed in medullary mitochondrial-enriched fractions, which are identical to the bands observed in the adrenal cortex (Fig. 3-5-1). The positive immunostaining of StAR protein has been observed in medulla sections (Lo et al, 1998). While the medulla sample is supposedly free from the contamination with the adrenal cortex, there may well be islands or infiltration of cortical cells into the medulla. On the other hand, StAR protein might play a role in medulla, which may be distinct to its role in adrenal cortex and gonads. This latter issue was not taken forward in this study.

3.5.3 Confirmation of StAR protein in subcellular fractions

To confirm StAR protein in subcellular fractions, the mitochondrial-enriched fractions (kindly prepared by the Dr. David Apps, Department of Biomedical Sciences, University of Edinburgh) from the adrenal cortex ZG and ZFR were analysed by Western blotting combined with neutralisation of peptide antigen. Three PVDF membrane strips were incubated in 10,000-fold diluted primary antibody without antigen (control) and with 10^{-7} mg/L and 10^{-5} mg/L antigen respectively. After washing off excess primary antibody and antigen, all strips were incubated with 100,000-fold secondary antibody. Clearly, the Western immunoblot on addition of 10^{-5} mg/L antigen showed that the protein bands from all fractions disappeared, while a Western immunoblot with 10^{-7} mg/L antigen revealed all the protein bands seen in the control strip (Fig. 3-5-3).

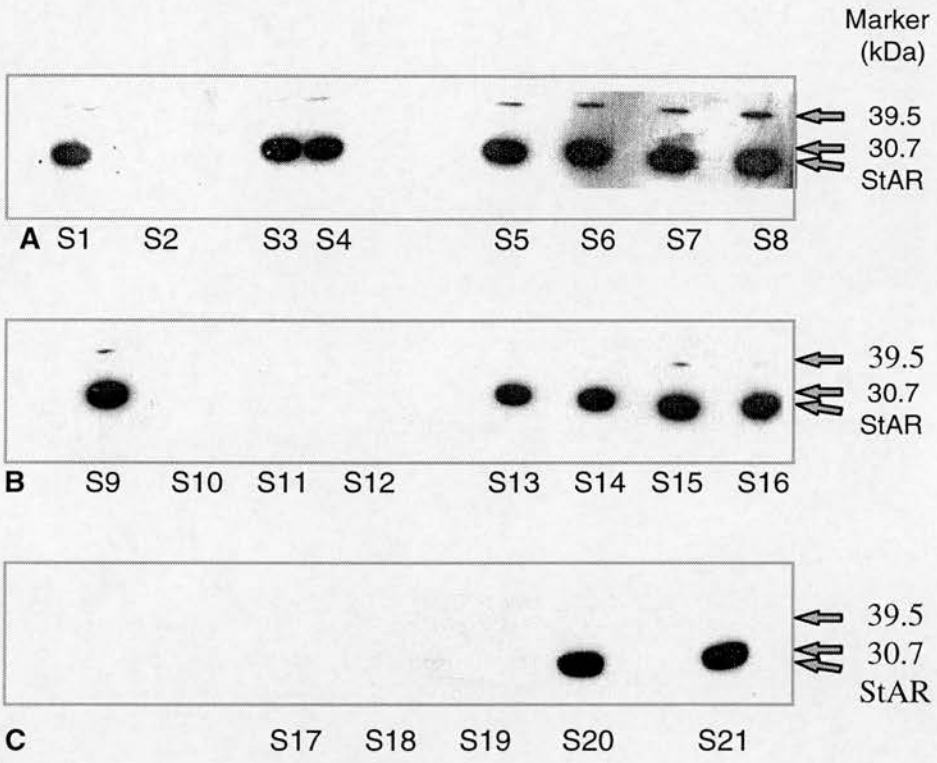

Fig. 3-4 Validation of sheep anti-bovine peptide StAR antibody· All samples (25 μg of total cellular protein from bovine cortex tissue) were resolved on 12.5% mini SDS-PAGE gels and blotted on to PVDF membranes. Each membrane was divided into four or five strips. Sheep anti-bovine peptide antibody (1:10,000) plus various concentrations of antigen and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS buffer containing 3% milk, then incubated with strips for 1 hr respectively. The strips of each group were combined together prior to the visualisation of StAR protein bands using a Pierce-ECL kit. Details of the experimental conditions (antigen concentration mg/L) are listed below:

Group A		Group B		Group C	
Strip no.	Antigen	Strip no.	Antigen	Strip no.	Antigen
S1	Control	S9	Control	S17	$3.0x10^{-6}$
S2	$1.0x10^{-5}$	S10	$1.0x10^{-5}$	S18	$2.5x10^{-6}$
S3	$1.0x10^{-7}$	S11	$6.0x10^{-6}$	S19	$2.0x10^{-6}$
S4	$1.0x10^{-9}$	S12	$3.0x10^{-6}$	S20	$1.5x10^{-6}$
S5	$1.0x10^{-11}$	S13	$1.0x10^{-6}$	S21	$1.0x10^{-6}$
S6	$1.0x10^{-13}$	S14	$6.0x10^{-7}$		
S7	$1.0x10^{-15}$	S15	$3.0x10^{-7}$		
S8	$1.0x10^{-17}$	S16	$1.0x10^{-7}$		

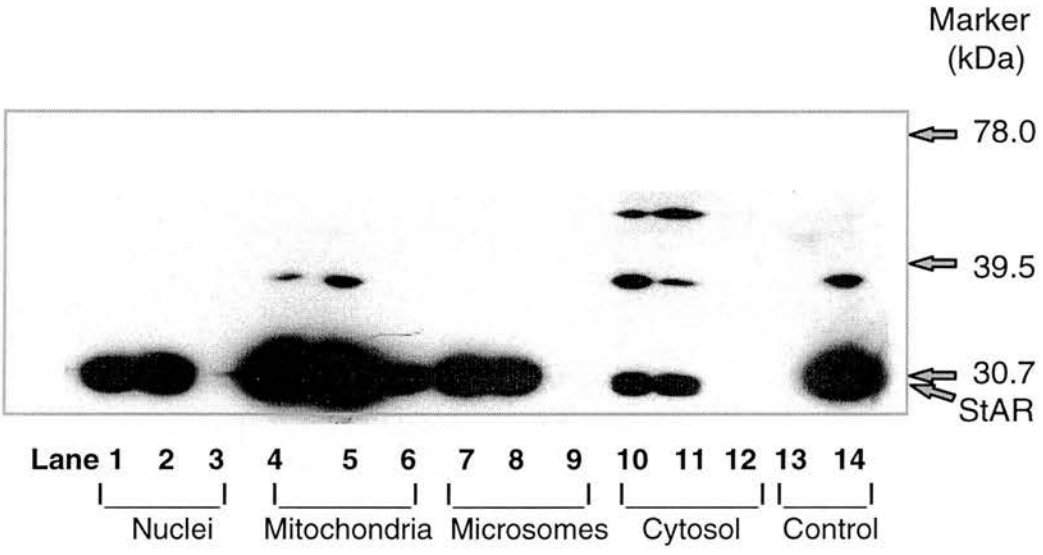

Fig. 3-5-1 StAR protein Western immunoblot of subcellular fractions of bovine adrenal cortex and medulla. Lanes 1, 4, 7 and 10: ZG; lanes 2, 5, 8 and 11: ZFR; lanes 3, 6, 9 and 12: medulla; lane 13: Negative control (bovine liver); lane 14: Positive control (bovine adrenal cortex). All samples (25 µg protein) were resolved on a 12.5% large SDS-PAGE gel and blotted to a PVDF membrane. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS buffer containing 3% milk. Protein bands were finally visualised using a Pierce-ECL kit.

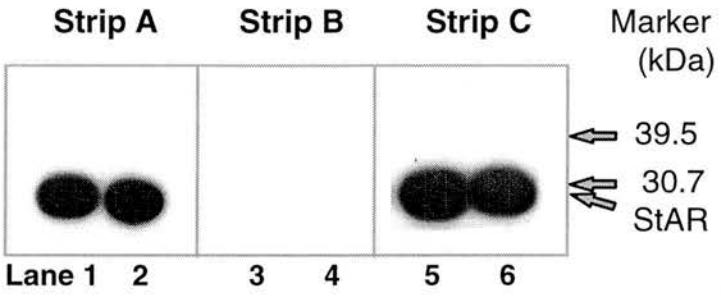

Fig. 3-5-3 StAR protein Western immunoblots of mitochondrial-enriched fractions of bovine adrenal tissues confirmed by antigen competition test. Strip A: Control group without antigen; strip B: Sheep anti-bovine antibody mixed with $1.0x10^{-5}$ mg/L peptide antigen; strip C: Sheep anti-bovine antibody mixed with $1.0x10^{-7}$ mg/L peptide antigen. Lanes 1, 3 and 5: Fractions of ZG; lanes 2, 4 and 6: Fractions of ZFR. All samples (25 μg of total cellular protein) were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. This membrane was blocked in 5% milk/PBS buffer overnight, then divided into three strips. Sheep anti-bovine peptide antibody (1:10,000) with or without different concentrations of antigen and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS buffer containing 3% milk, then incubated with strips for 1 hr respectively. Protein bands were finally visualised using a Pierce-ECL kit.

3.6 COMPARISON OF ANTI-PEPTIDE BOVINE StAR ANTIBODY AND ANTI-RECOMBINANT MOUSE StAR ANTIBODY

Western immunoblot using the anti-peptide antibody produced very strong doublet StAR bands (approx. 28-30 kDa) with some possibly StAR protein-associated bands (approx. 34, 40 and 50 kDa) (Fig. 3-6-1&2). Anti-recombinant StAR antibody only revealed the doublet bands around 28-30 kDa, suggesting its specificity may be better than that of peptide antibody (Fig. 3-6-3&4). However, it also gave a more unsatisfactory background. Although the background might be reduced or removed by purifying the antibody, the signal appeared not strong enough for analysis on the Bio-Rad image system (Fig. 3-6-4). This would therefore pose a problem for quantification of the low basal level of StAR protein in cultured untreated cells. Moreover, our very limited supply of this polyclonal antibody also precluded any further detailed study. Generally, the 28-30 kDa doublet bands of StAR protein appeared only as a single band on mini SDS-PAGE gels due to the limited resolution of the mini-gel.

3.7 DETECTION OF StAR PROTEIN IN BOVINE OVARY

StAR protein was also observed in bovine ovarian theca tissue and granulosa cells in which StAR protein levels were much lower compared with those in the adrenal cortex tissue and cells (Fig. 3-7-1). More importantly, StAR protein was revealed in 6-day cultured untreated bovine theca cells, but at an extremely low level. The levels of StAR protein were markedly increased with bovine luteinizing hormone treatment (Fig. 3-7-2).

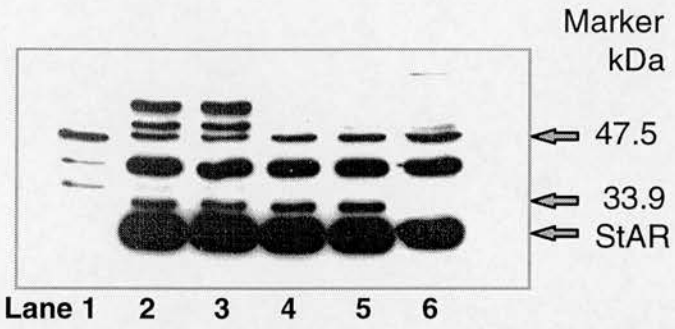

Fig. 3-6-1 StAR protein Western immunoblot using anti-peptide StAR antibody. Lane 1: Bovine liver tissue; lane 2 and 3: Bovine adrenal cortex tissue; lane 4 and 5: Freshly isolated bovine adrenal ZF cells; lane 6: 3-Day cultured adrenal ZF cells. All samples (25 μg of total cellular protein) were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in Pierce blocking buffer overnight. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk, then incubated with strips for 1 hr respectively. The membrane was washed with PBS for 45 min before and after applying antibodies. Protein bands were finally visualised using a Pierce-ECL kit.

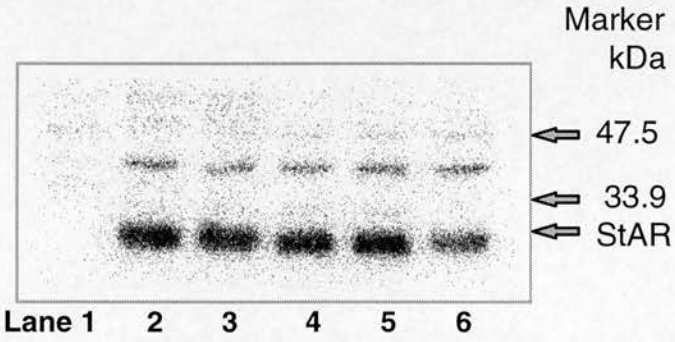

Fig. 3-6-2 StAR protein Western immunoblot using anti-peptide StAR antibody (Bio-Rad image print of Fig. 3-6-1).

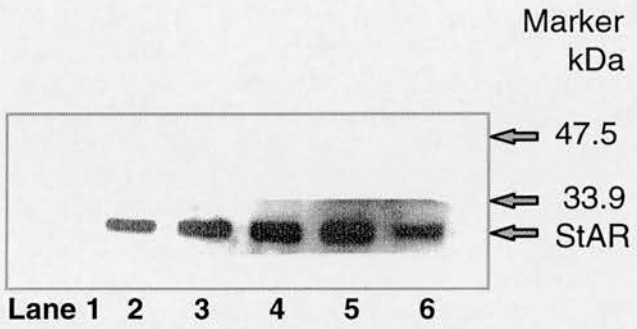

Fig. 3-6-3 StAR protein Western immunoblot using anti-recombinant StAR antibody. Lane 1: Bovine liver tissue; lane 2 and 3: Bovine adrenal cortex tissue; lane 4 and 5: Freshly isolated bovine adrenal ZF cells; lane 6: 3-Day cultured adrenal ZF cells. All samples (25 μg of total cellular protein) were resolved on a 12.5% mini SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in Pierce blocking buffer overnight. Rabbit anti-mouse recombinant StAR antibody (1:5,000) and donkey anti-rabbit antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 1% milk, then incubated with membrane for 1 hr respectively. The membrane was washed with PBS buffer for 45 min before and 2 hr after applying antibodies. Protein bands were finally visualised using a Pierce-ECL kit.

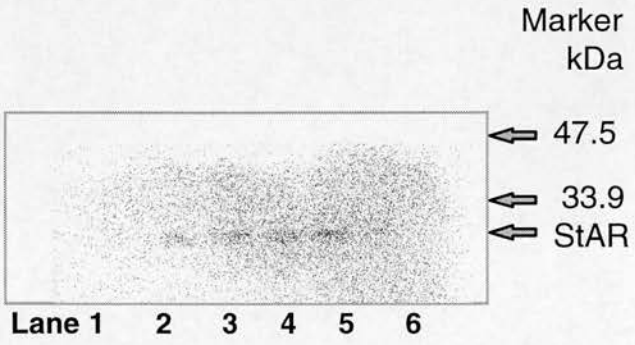

Fig. 3-6-4 StAR protein Western immunoblot using anti-recombinant StAR antibody (Bio-Rad image print of Fig. 3-6-3).

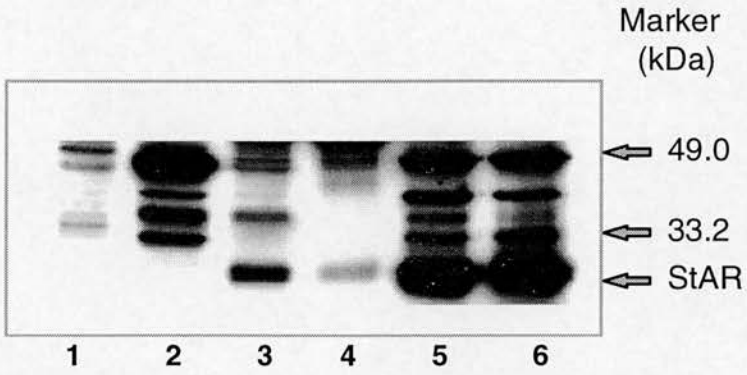

Fig. 3-7-1 StAR protein Western immunoblot of various bovine tissues. Lane 1: COS-1 cells (negative control); lane 2: Liver tissue (negative control); lane 3: Ovarian theca tissue; lane 4: Ovarian granulosa cells; lane 5: adrenal cortex tissue (positive control) and lane 6: Freshly isolated adrenal ZF cells. All samples (25 μg of protein) were resolved on a 12.5% mini SDS-PAGE gel. Sheep anti-bovine peptide antibody (1:7,500) and donkey anti-sheep/goat antibody conjugated with HRP (1:10,000) were diluted in PBS/10% Pierce blocking buffer containing 1% milk.

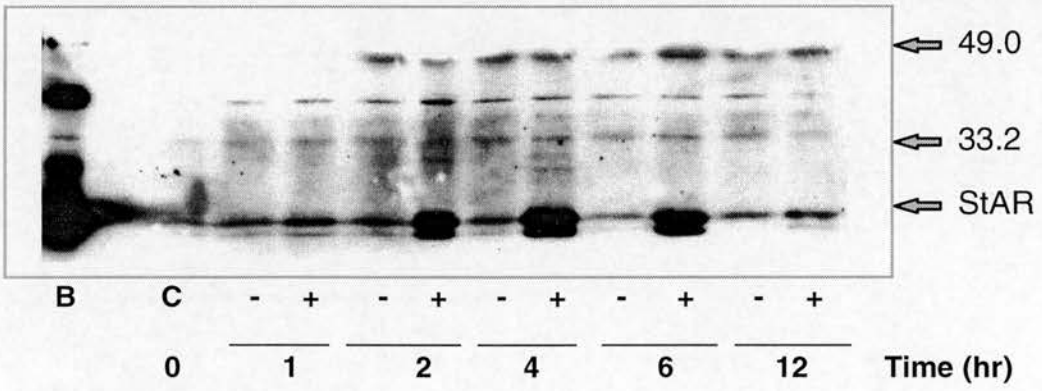

Fig. 3-7-2 StAR protein Western immunoblot in cultured bovine ovarian theca cells in the absence and presence of 10 ng/ml LH. B: Adrenal cortex tissue (positive control); C: Zero time point untreated cells. All samples (15 μg of sample B and 25 μg of all others) were resolved on a 12.5% large SDS-PAGE gel. Sheep anti-bovine peptide antibody (1:7,5000) and donkey anti-sheep/goat antibody conjugated with HRP (1:10,000) were diluted in PBS/10% Pierce blocking buffer containing 1% milk.

3.8 DISCUSSION

The optimisation of the Western immunoblot methodology was taken through four stages. Firstly, the evaluation of colour detection indicated that this method was impractical for quantification due to transient and insensitive signals. Secondly, the Amersham ECL kit was employed, which worked well for X-OMAT film. However, the signal produced by this reagent was very poorly detected by the Bio-Rad image analysis system, i.e. too weak for quantification. This was probably because the wavelength of light emission did not exactly match with the sensitive range of the chemiluminescence screen. Although there are some other analyses for Western immunoblot quantification, the Bio-Rad image system is the one currently available in our laboratory. Therefore, the utility of another ECL product made by Pierce Ltd. was explored and found to be of value in obtaining quantitative results.

The working conditions of Western immunoblot using the Pierce ECL kit were optimised and they were suitable for both X-OMAT and Bio-Rad image system. However, the ideal conditions (particularly the antibody dilution buffer) for X-OMAT film were not consistent with those of Bio-Rad image system. Thus, the conditions were further modified to meet the requirement for accurate quantification. The signals were intensified again in a combined PBS/10% Pierce blocking buffer for antibody dilution, permitting the quantification of weak signals produced by conventional Western blotting. Pierce blocking buffer appears to have at least two functions: blocking the non-specific banding and enhancing the signal (presumably the enhancing principle of this buffer is different from that of ECL detection reagent). The strong positive correlation between the volume counts (integrated signal intensity) and protein concentration demonstrated that newly established method was linear, reproducible and sensitive for quantification of StAR protein.

In fact, sometimes working conditions needed to be re-evaluated when a new batch of reagent was used as there were subtle variations among different batches products (the reason remains unknown and unresolved as the P-ECL kit is a patented product). The recommended starting point for StAR protein Western immunoblot are 10,000-fold dilution for primary antibody and 100,000-fold for secondary antibody dilution in 3% milk PBS buffer or 10% Pierce blocking buffer. In

general, an appropriate detection system can be established empirically for the proteins of interest.

The optimisation of Western immunoblot conditions has provided an efficient approach to observe and quantify StAR protein. Importantly, the validation data for the primary antibody demonstrated the specificity of the sheep anti-bovine peptide StAR antibody. Western immunoblot of subcellular fractions also proved that mature StAR protein predominated in mitochondrial-enriched fractions. Moreover, this detection method allowed visualisation of StAR protein in bovine ovarian theca and granulosa cells that expressed StAR protein at very low levels. These are suggestive that sheep anti-bovine peptide StAR antibody as a primary antibody is well suitable for Western immunoblot analysis.

Western immunoblot of large SDS-PAGE gels demonstrate clear doublet bands of StAR band at 28-30 kDa (appear as a single band in a mini-gel), which is consistent with previous work on bovine adrenal ZF cells (Nishikawa et al, 1996) and ZG cells (Cherradi et al, 1997). The doublet may represent the protein species derived from one core protein with different cleavage or phosphorylation proteins. This requires to be clarified by 2-D electrophoresis. A band closed to the 39.5 kDa marker was demonstrable and neutralised by peptide antigen competition, suggesting that it may be the precursor form of StAR protein. However, it did not appear using anti-recombinant mouse StAR antibody. Further investigation is required to clarify its properties.

CHAPTER 4 EXPRESSION OF StAR PROTEIN, CORTISOL SECRETION AND cAMP PRODUCTION IN RESPONSE TO VARIOUS HORMONAL TREATMENTS

4.1 INTRODUCTION

4.1.1 ACTH-regulated steroidogenesis

Steroidogenesis is positively or negatively regulated by a number of agents. It is well established that ACTH has an important role in the regulation of steroid production in adrenal zona fasciculata cells. The actions of ACTH can be divided into two phases on a temporal basis. Acute (short-term) responses occur within seconds to minutes and chronic (long-term) responses require hours/days. The acute effect is to mobilise and deliver cholesterol, the precursor of all steroid hormones, to the mitochondrial inner membrane. On the other hand, the chronic effect involves regulation in transcription of genes that encode steroidogenic enzymes, maintaining optimal glucocorticoid productive capacity (Jefcoate et al., 1987; Simpson and Waterman, 1988). The chronic effects may involve changes in the levels of CYP11A, CYP11B1, CYP17, CYP21B and 3β-HSD.

The primary event of acute ACTH action is an intramitochondrial step to translocate cholesterol. Early studies established that a labile protein appeared to facilitate the translocation of cholesterol to the inner mitochondrial membrane where the first enzymic reaction of steroid synthesis occurs (Stone and Hechter, 1954). After several decades of investigation, StAR protein has been recognised as a most promising candidate to fulfil this task. The essential role of StAR in adrenal steroidogenesis has been exemplified by studies of StAR-null mice and humans with a dysfunctional StAR gene, which demonstrated that the substantial decrease in the conversion of cholesterol to pregnenolone was clearly associated with dysfunctional StAR protein (Kallen et al., 1998a). Both biochemical and genetic findings are indicative that StAR is a key regulator in controlling cholesterol transfer in the adrenal cortex.

4.1.2 Angiotensin II-regulated steroidogenesis

Angiotensin II (Ang II) is another major hormonal regulator of steroid synthesis in the adrenal glands. The principal function of Ang II on steroidogenesis is mineralocorticoid production in adrenal ZG through IP_3 and Ca^{2+} signalling pathway (Rossier, 1997).

In some species Ang II also stimulates cortisol production in the adrenal ZF. In bovine adrenal ZF cells, Ang II-induced cortisol secretion may be mediated by depolarization-dependent Ca^{2+} entry and through inhibition of a novel potassium channel (I_{AC}). The mechanism for this steroidogenesis is distinct from that involving IP_3-triggerred Ca^{2+} release from intracellular stores (Mlinar et al., 1995). Other evidence has demonstrated that Ang II is able to stimulate cAMP formation in a concentration and time-dependent manner with a significant increase at 10^{-9} M Ang II and a maximal response at 10^{-7} M Ang II. A subtype of the Ang II receptor is responsible for stimulating cAMP production and steroidogenesis in bovine adrenal ZF cells (Rainey et al., 1991). Furthermore, Ang II significantly augments the cAMP levels induced by ACTH. Potentiation of ACTH-induced cAMP production by Ang II was also observed in COS-7 cells which were transiently expressing the AT_{1a} receptor, indicating that this combined effect of Ang II and ACTH on the increase in cAMP formation may be a more general biochemical process (Baukal et al., 1994).

The parallel time courses of StAR protein and aldosterone production were indicative that Ang II (10^{-8} M)-induced steroid synthesis was correlated with the increase in the expression of StAR protein in a temporally co-ordinated manner in H295R human adrenocortical carcinoma cells (Clark et al., 1995b). On the other hand, the question of whether Ang II-induced steroidogenesis is regulated by StAR protein in bovine ZF cells has not been addressed.

4.1.3 Aims of this study

Although ACTH-induced steroidogenesis in bovine ZF cells has been extensively studied *in vitro*, most attention has focused on the effects of pharmacological concentrations of ACTH and the more chronic response. The regulation of

steroidogenesis acutely and at concentrations of ACTH that are profoundly important in terms of physiological implications has yet to be more clearly defined.

The purposes of this study were:

- To obtain information on the abundance of StAR protein in the absence and presence of ACTH (10^{-8} M) over a five-day cell culture period.
- To determine an appropriate treatment starting time and effector concentrations for the further study of StAR protein expression, steroidogenesis and signalling pathways.
- To establish the cell responsiveness to various concentrations of ACTH in both acute and chronic phases with respect to the temporal relationship of StAR protein, cortisol secretion and cAMP production.
- To evaluate the integrative effects of ACTH and Ang II on StAR protein and cortisol levels in bovine adrenal ZF cells.

4.2 CHANGES OF StAR PROTEIN LEVELS WITH ACTH TREATMENT ON DIFFERENT DAYS OF CULTURE

The effect of 6 and 24 hr ACTH (10^{-8} M) treatment schedules on StAR protein levels after a culture period of 0-4 days as demonstrated on a Western immunoblot is shown in Fig. 4-2-1a. After quantification these data are shown in Fig. 4-2-1b, the open columns indicate the abundance of StAR in untreated cells, demonstrating the initial level declining to a stable lower level after two days of culture, whereas the solid columns showed treated cells in response to ACTH (10^{-8} M). The levels of StAR protein were high at 6 hr on Day 0 in both untreated and treated cells and distinct from the basal levels after ACTH treatment at 6 hr on Day 2 and Day 3 of culture.

Fig. 4-2-2 illustrates that the levels of StAR protein increased with respect to the basal at 6 hr in the presence of 10^{-8} M ACTH from Day 0 to Day 4. In general, the cell responsiveness to ACTH at 6 hr was greater than that at 24 hr. StAR protein showed a marked change (5.1 ± 1.8 fold, $P<0.05$) after 6 hr treatment on Day 2 and Day 3 (3.9 ± 1.3 fold, $P<0.05$).

According to these observations, cells on both Day 2 and Day 3 were clearly responsive to ACTH treatment, i.e. the culture periods were best suited for subsequent experiments. As cells need to be serum-deprived overnight in order to minimise the interference of serum components on cortisol secretion, this work was conducted by initiating cell treatment on Day 3 of culture.

4.3 CONCENTRATION RESPONSE CURVES OF StAR PROTEIN LEVELS, CORTISOL SECRETION AND cAMP PRODUCTION

To evaluate the effect of various concentrations of ACTH on the expression of StAR protein, cortisol secretion and cAMP formation, cells were treated with 10^{-14} to 10^{-7} M ACTH starting on Day 3 of culture. The media and cells were collected after 1 and 6 hr treatments in this study.

Fig 4-3-1a&b show the levels of StAR protein established by Western immunoblot in response to various concentrations of ACTH. At 1 hr StAR protein levels were slightly changed at 10^{-12}-10^{-7} M ACTH. At 6 hr the expression of StAR protein

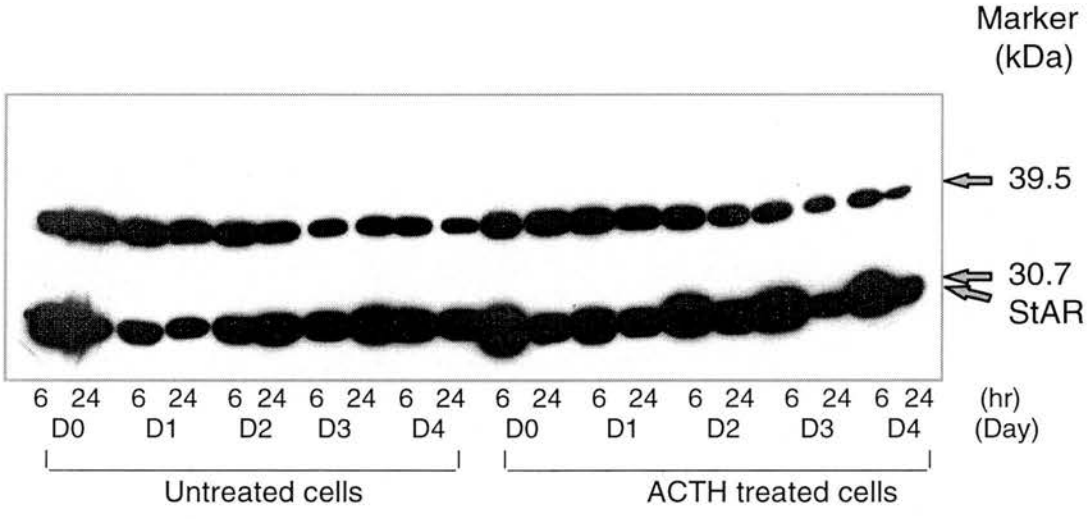

Fig. 4-2-1a Western immunoblot of StAR protein in response to ACTH treatment D0: Day 0; D1: Day 1; D2: Day 2; D3: Day 3 and D4: Day4. Cells were treated with and without ACTH (10^{-8} M) independently on each day and harvested after 6 and 24 hr culture. All samples (25 µg protein) were resolved on a 12.5% large SDS-PAGE gel and blotted on to a PVDF membrane. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS/10% Pierce blocking buffer containing 0.5% milk.

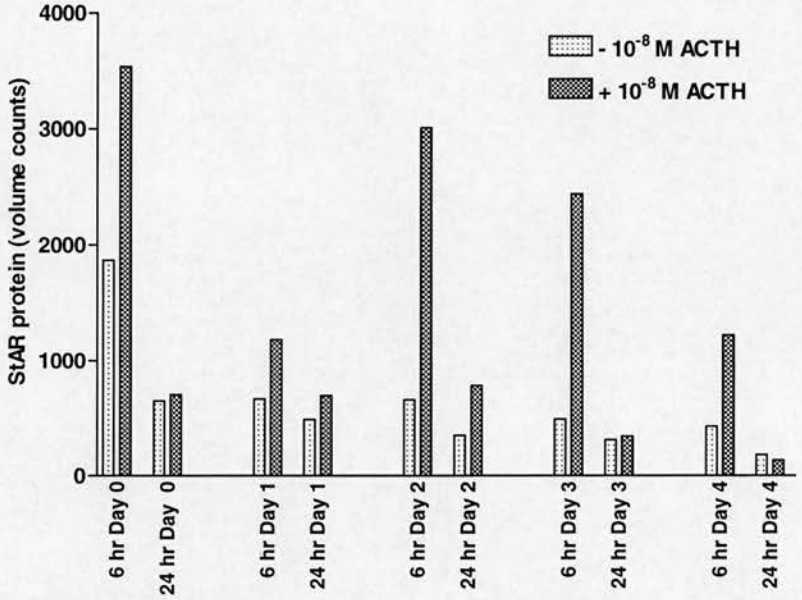

Fig. 4-2-1b StAR protein levels in response to ACTH treatment. Cells were treated with ACTH (10^{-8} M) on each day and collected at 6 and 24 hr time points. StAR protein bands were quantified using Bio-Rad image analysis. A representative single experiment is shown.

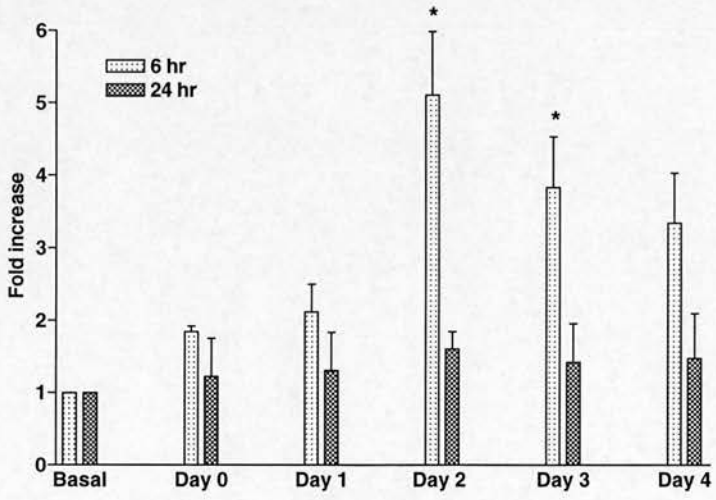

Fig. 4-2-2 Changes of StAR protein levels in response to ACTH treatment. Cells were treated with and without ACTH (10^{-8} M) on each day independently and collected at 6 and 24 hr time points. StAR protein bands were quantified using Bio-Rad image analysis. Values from four experiments are expressed as mean ± SEM (treated over untreated value at each time point), n=4. Data were tested by ANOVA, followed by Dunnett multiple comparison (*$P<0.05$).

increased with the incremental ACTH concentrations, starting from 10^{-12} M ACTH and attaining to high levels at 10^{-10} -10^{-7} M ACTH.

In addition, after 6 hr ACTH (10^{-10}-10^{-8} M) treatment, a minor band appeared with a size about 34 kDa. This may represent a newly synthesised precursor of StAR protein; it appeared to have a unstable character.

Fig. 4-3-2 demonstrated that after 1 and 6 hr of treatment cortisol levels were remarkably elevated when cells were treated with 10^{-12} M ACTH compared with 10^{-14} and 10^{-13} M ACTH. Further increases occurred at 10^{-11} M ACTH, plateauing at 10^{-10}-10^{-8} M ACTH.

As shown in Fig. 4-3-3, the changes in cAMP were hardly detectable at 10^{-14}-10^{-12} M ACTH but elevated at 10^{-11} M ACTH and reached maximal levels at 10^{-9}-10^{-7} M ACTH at both 1 and 6 hr. The detailed changes of all above treatments are summarised in Table 4.3.

Table 4.3 A representative concentration response experiment performed in triplicate. Values are expressed as fold increase (mean ± SEM)

Incubation	1 hr			6 hr		
[ACTH]	StAR	Cortisol	cAMP	StAR	Cortisol	cAMP
10^{-14} M	1.1 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	0.6 ± 0.1
10^{-13} M	1.1 ± 0.3	1.2 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	0.6 ± 0.03
10^{-12} M	1.2 ± 0.1	17.2 ± 0.4	1.4 ± 0.1	1.5 ± 0.3	12.6 ± 0.7	1.6 ± 0.1
10^{-11} M	1.4 ± 0.2	38.3 ± 3.3	50.1 ± 6.9	2.7 ± 0.5	50.7 ± 2.8	339.7±29.0
10^{-10} M	1.2 ± 0.2	38.2 ± 1.2	233.9±11.4	3.8 ± 0.8	89.1 ± 4.6	347.7±34.5
10^{-9} M	1.2 ± 0.2	39.1 ± 2.5	275.5±8.2	3.9 ± 0.7	84.8 ± 3.8	501.5±3.7
10^{-8} M	1.2 ± 0.2	66.9 ± 1.9	348.3±13.3	3.6 ± 0.4	106.1 ± 3.3	560.9±32.5
10^{-7} M	1.2 ± 0.2	64.2 ± 7.3	346.6±30.0	3.1 ± 0.4	95.4 ± 3.7	498.0±21.0

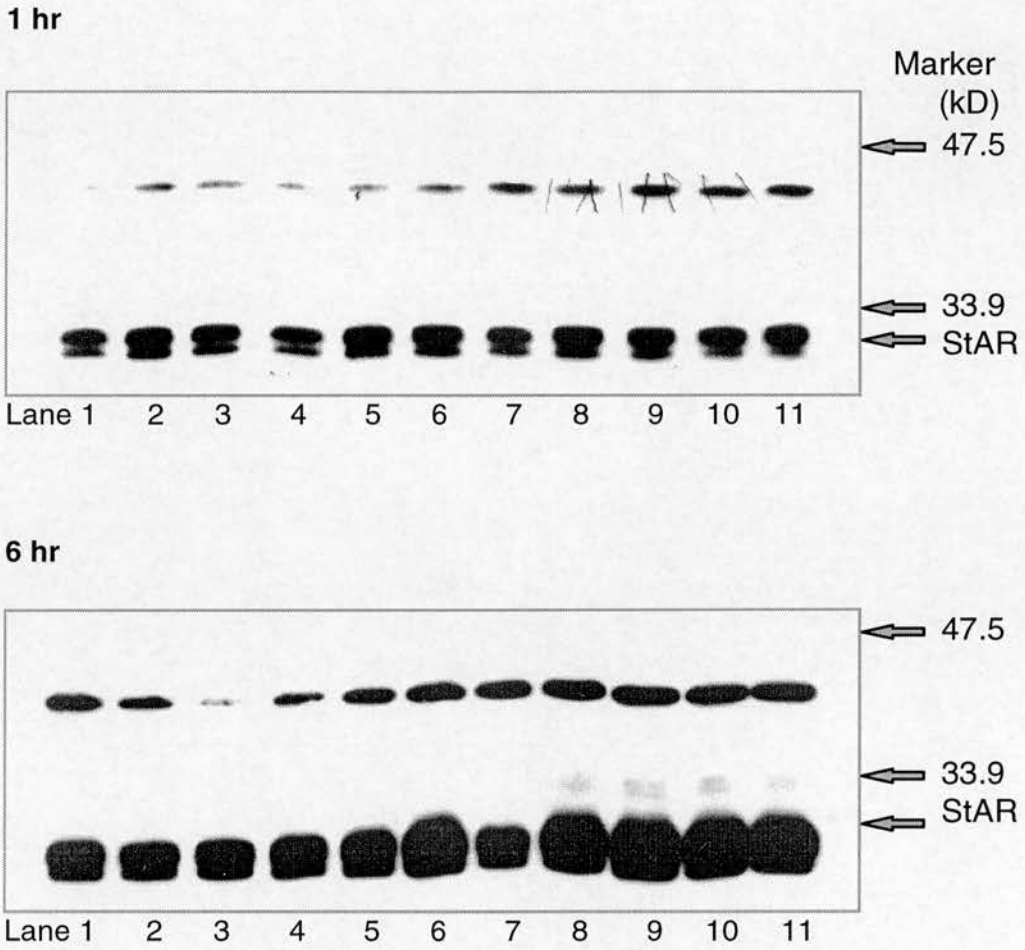

Fig. 4-3-1a Western immunoblot of StAR protein in response to various concentrations of ACTH at 1 hr (upper panel) and at 6 hr (lower panel). Lane 1 and 7: Zero time point control; Lane 2: 1 hr or 6 hr untreated cells; Lane 3: ACTH (10^{-14} M) treated cells; Lane 4: ACTH (10^{-13} M) treated cells; Lane 5: ACTH (10^{-12} M)treated cells; Lane 6: ACTH (10^{-11} M) treated cells; Lane 8: ACTH (10^{-10} M) treated cells; Lane 9: ACTH (10^{-9} M) treated cells; Lane 10: ACTH (10^{-8} M) treated cells; Lane 11: ACTH (10^{-7} M) treated cells. Samples (25 µg protein) were resolved on two 12.5% large SDS-PAGE gels. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1: 25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

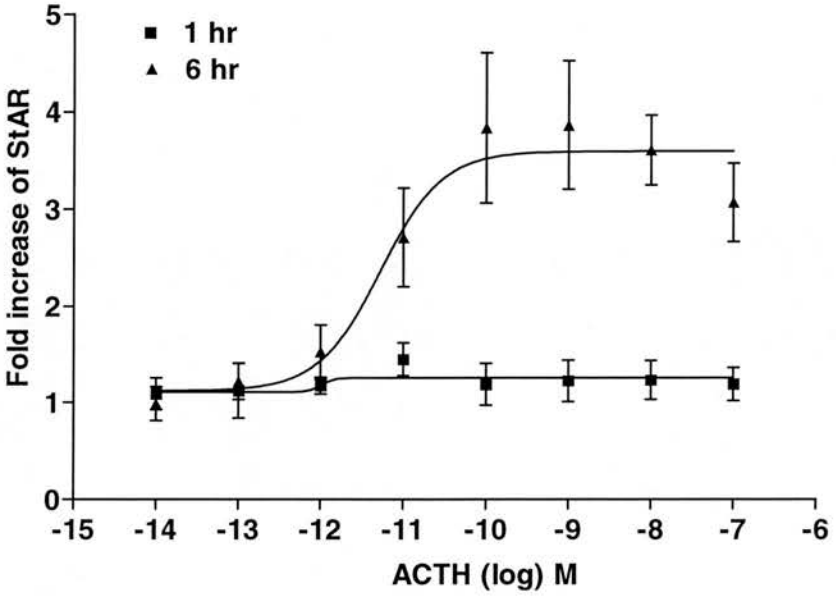

Fig. 4-3-1b Concentration response curves for StAR protein in response to various concentrations of ACTH. Cells were treated with ACTH ranging from 10^{-14} M to 10^{-7} M and collected for Western blotting after 1 hr and 6 hr treatment. Values are expressed as fold increase over the basal level (mean ± SEM), n=3. An experiment conducted in triplicate is shown.

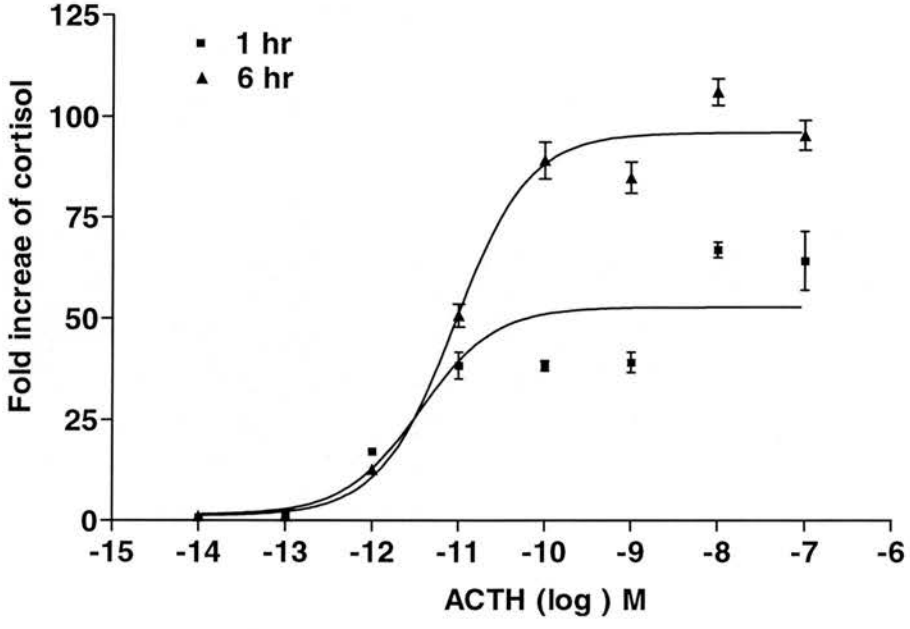

Fig. 4-3-2 Concentration response curves for cortisol levels in response to various concentrations of ACTH. Cells were treated with ACTH ranging from 10^{-14} M to 10^{-7} M and the media were collected for cortisol after 1 hr and 6 hr treatment. Values are expressed as fold increase over the basal level (mean ± SEM), n=3. Data were obtained from the same experiment as Fig. 4-3-1b.

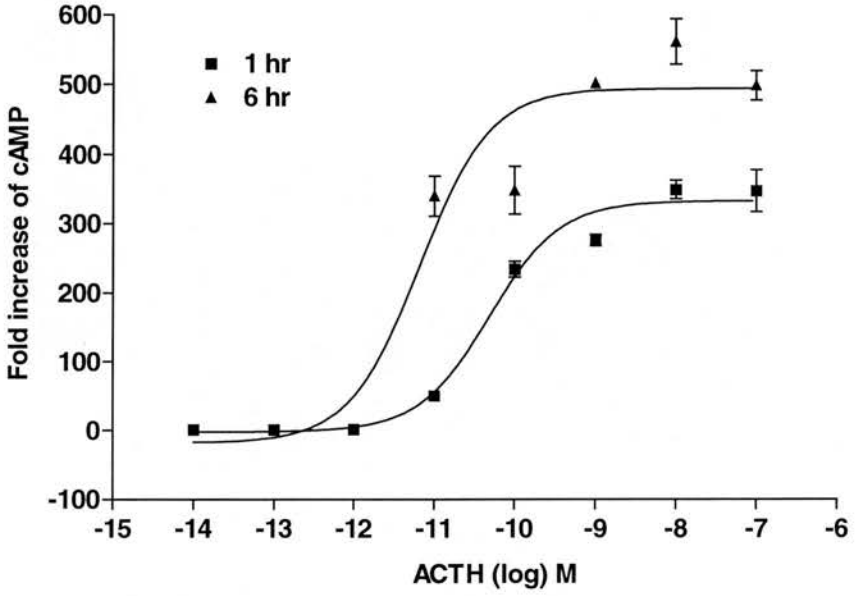

Fig. 4-3-3 Concentration response curves for cAMP levels in response to various concentrations of ACTH. Cells were treated with ACTH ranging from 10^{-14} M to 10^{-7} M and the media were collected for cAMP assay after 1 hr and 6 hr treatments. Values are expressed as fold increase over the basal level (mean ± SEM), n=3. Data were obtained from the same experiment as Fig. 4-3-1b.

At 1 hr the changes of StAR protein were poorly correlated with the elevated cortisol level ($r=0.14$, $P>0.05$; Fig. 4-3-4a). However, at 6 hr the StAR protein level was strongly correlated with the cortisol levels ($r=0.76$, $p<0.0001$; Fig. 4-3-4b).

There was no correlation between the increase of StAR protein versus cAMP at 1 hr ($r=0.16$, $P>0.05$; Fig. 4-3-4c). In contrast, the changes of StAR protein were closely correlated with the cAMP levels at 6 hr ($r=0.77$, $P<0.0001$; Fig. 4-3-4d).

The cortisol levels were tightly coupled to the elevation of cAMP at both 1 hr ($r=0.82$, $p<0.0001$; Fig. 4-3-4e) and 6 hr ($r=0.91$, $P<0.0001$; Fig. 4-3-4f).

The effects of ACTH on the expression of StAR protein and cortisol secretion were time- and concentration-dependent over the range of 10^{-14}-10^{-7} M ACTH. The profile of changes in StAR protein at 6 hr were different from that at 1 hr. In the acute phase (1 hr), although cAMP levels were rapidly increased, the levels of StAR protein were not significant, this may be due to the delayed newly synthesised protein from StAR mRNA. At relatively chronic phase (6 hr) the cortisol levels were dramatically elevated with more expression of StAR protein at 10^{-11}-10^{-7} M ACTH, in parallel with the increase in cAMP production. The profiles for cortisol and cAMP concentration response curves ranging from 10^{-11}-10^{-7} M ACTH were very similar to those at both 1 and 6 hr, suggesting that cAMP was a major second messenger.

On the basis of the concentration response experiment, 10^{-8} M ACTH was selected as representative of high concentration (pharmacological or supraphysiological level) and 10^{-12} M ACTH as representative of lower, perhaps a more physiological concentration for treatments.

4.4 EFFECTS OF HIGH-CONCENTRATION ACTH (10^{-8} M) TREATMENT ON THE LEVELS OF StAR PROTEIN, CORTISOL AND cAMP

To understand further the relationship between the expression of StAR protein and steroidogenesis the effects of high-concentration ACTH treatment were evaluated in more detail. The more detailed changes of StAR protein levels, cortisol and

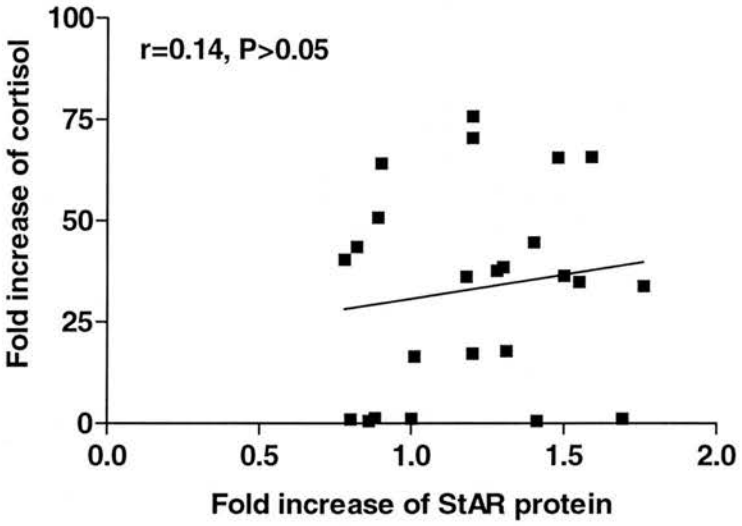

Fig. 4-3-4a Correlation between the levels of StAR protein and cortisol for concentration (10^{-14} - 10^{-7} M ACTH) response curve at 1 hr.

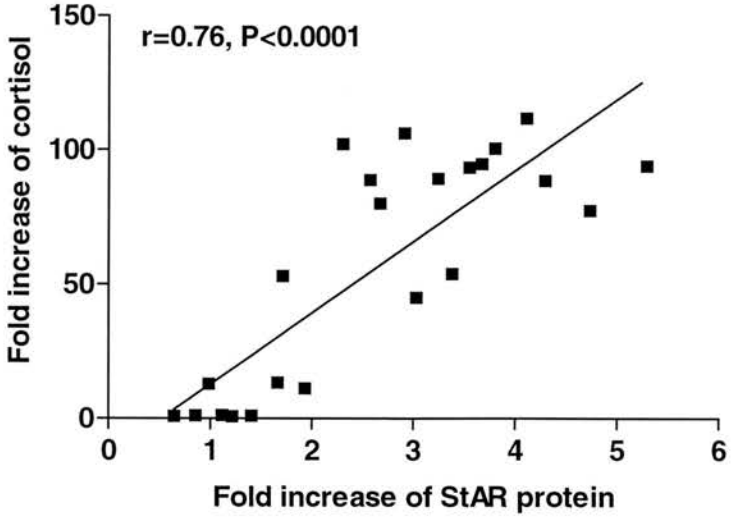

Fig. 4-3-4b Correlation between the levels of StAR protein and cortisol for concentration (10^{-14} - 10^{-7} M ACTH) response curve at 6 hr.

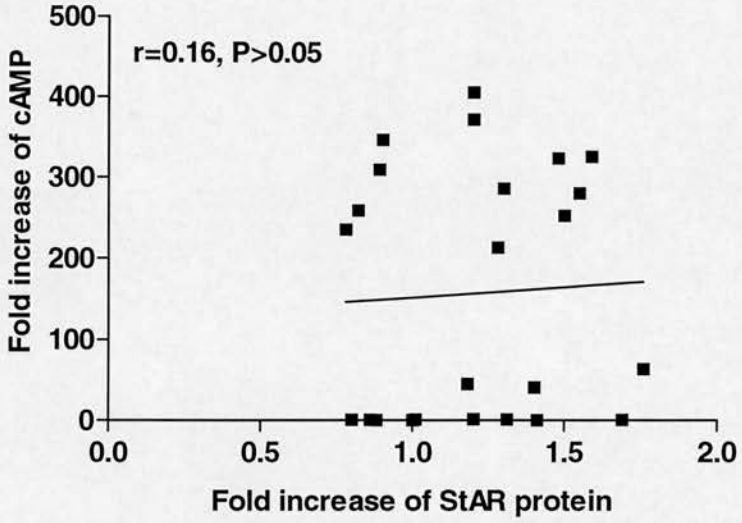

Fig. 4-3-4c Correlation between levels of StAR protein and cAMP for concentration (10^{-14} -10^{-7} M ACTH) response curve at 1 hr.

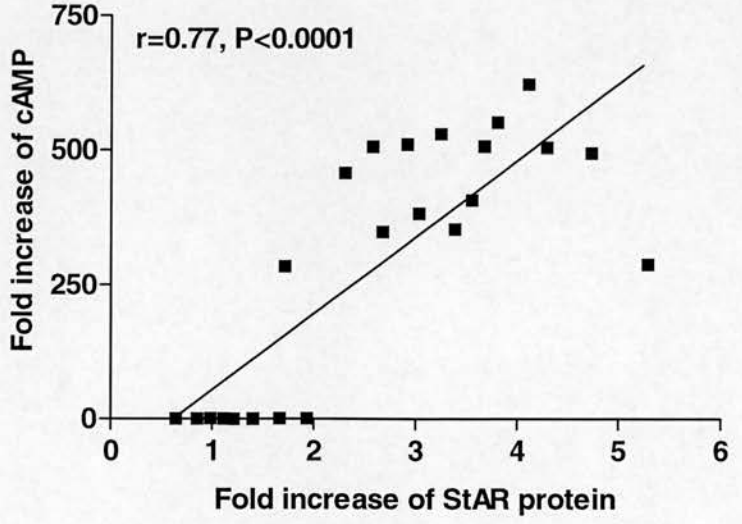

Fig. 4-3-4d Correlation between levels of StAR protein and cAMP for concentration (10^{-14} -10^{-7} M ACTH) response curve at 6 hr.

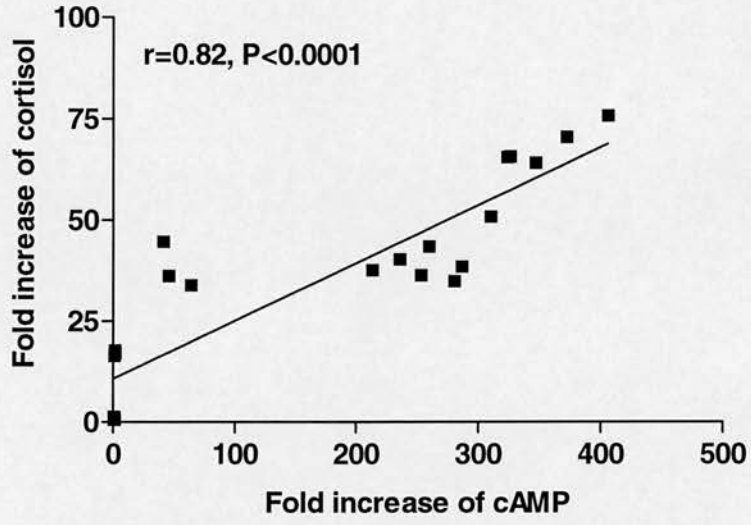

Fig. 4-3-4e Correlation between levels of cAMP and cortisol for concentration (10^{-14} –10^{-7} M ACTH) response curve at 1 hr.

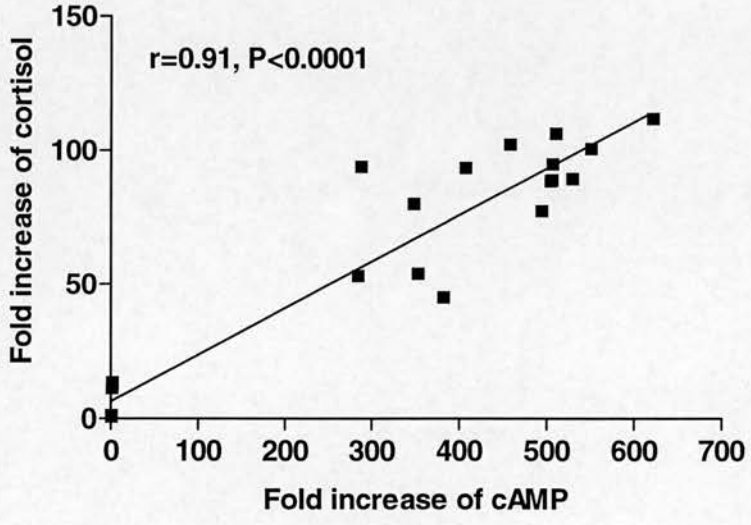

Fig. 4-3-4f Correlation between levels of cAMP and cortisol for concentration (10^{-14} –10^{-7} M ACTH) response curve at 6 hr.

cAMP in response to ACTH (10^{-8} M) were established in a 0.5-24 hr time course including acute, intermediate and chronic phases.

Fig 4-4-1a shows a Western immunoblot for a high-concentration ACTH time course. StAR protein significantly increased at 4 hr (3.9 ± 1.1 fold) and was maintained above the basal levels until 12 hr. The changes of StAR protein at 4, 6 and 8 hr were significantly different from those at 0.5, 1, 2 12 and 24 hr ($P<0.05$), and values at 2 and 4 hr were ambiguous between low and high levels (Fig. 4-4-1b).

Cortisol output increased dramatically at 1 hr and reached a peak (144.0 ± 36.6 fold) at 4 hr and declined after 12 hr incubation. The level of cortisol at 0.5 hr was significantly different from that at 24 hr ($P<0.05$) and also 2, 4, 6, 8 and 12 hr ($P<0.001$ for 0.5 hr versus four time points respectively). The level of cortisol at 1 hr was different from that at 0.5, 2, 4, 6, 8 hr ($P<0.05$ for 1 hr versus five time points respectively). There was no marked changes among the levels at 2, 4, 6 and 8 hr (Fig. 4-4-2).

The level of cAMP production at 0.5 hr was different from those at 1 hr ($P<0.05$), 2 hr ($P<0.01$), 4, 6 8, 12 and 24 hr ($P<0.001$); the level at 1 hr was different from those levels at 4, 6, 8, 12 and 24 hr ($P<0.001$); and the level at 4 hr was different from that at 6 hr ($P<0.05$), but not those at 8, 12 and 24 hr (Fig. 4-4-3).

High-concentration ACTH treatment resulted in a strongly correlated relationship between the expression of StAR protein and the production of cortisol ($r=0.81$, $P<0.0001$; Fig. 4-4-4), indicating that StAR protein may be a key factor for regulation of steroidogenesis.

4.5 EFFECTS OF LOW-CONCENTRATION ACTH (10^{-12} M) TREATMENT ON THE LEVELS OF StAR PROTEIN, CORTISOL AND cAMP

To evaluate the expression of StAR, cortisol secretion and cAMP production in response to ACTH (10^{-12} M), i. e. a concentration within the physiologic range, cells were treated over the same time course as that used for high-concentration ACTH (10^{-8} M) treatment.

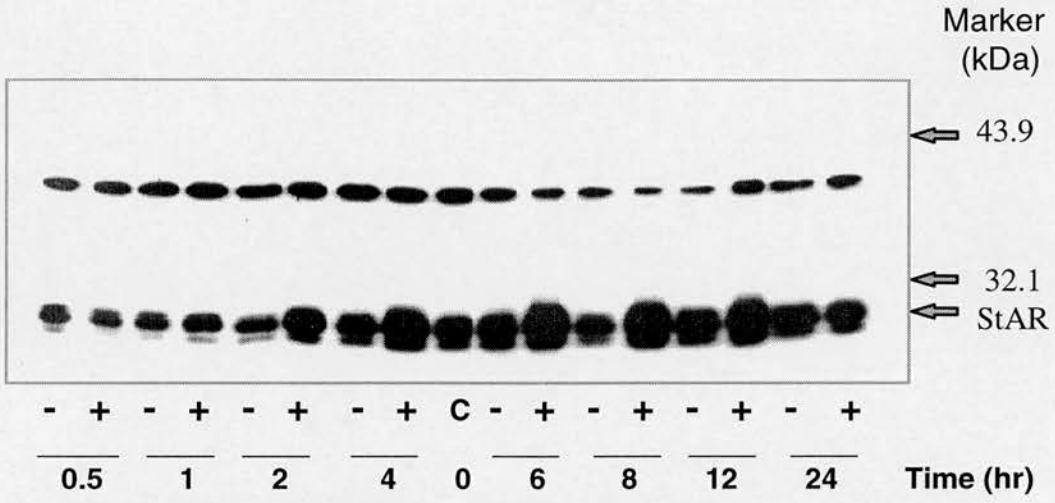

Fig. 4-4-1a Western immunoblot of StAR protein after high-concentration ACTH treatment. "-": Untreated cells; "+": ACTH (10^{-8} M) treated cells; C: Zero time point cells for control. All samples (25 μg protein) were resolved on a 12.5 % SDS-PAGE gel. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

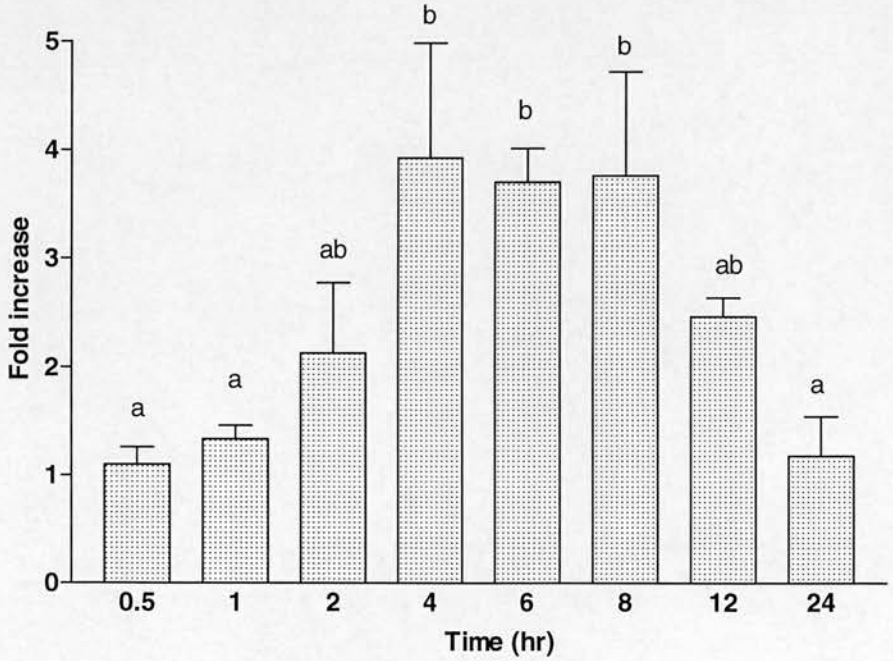

Fig.4-4-1b Changes of StAR protein levels for high-concentration ACTH time course. Cells were treated with ACTH (10^{-8} M) and cells were harvested at various time points. Values from three independent experiments are expressed as fold increase over the corresponding basal level (mean ± SEM), n=3. Columns without the same superscript letter were significantly different.

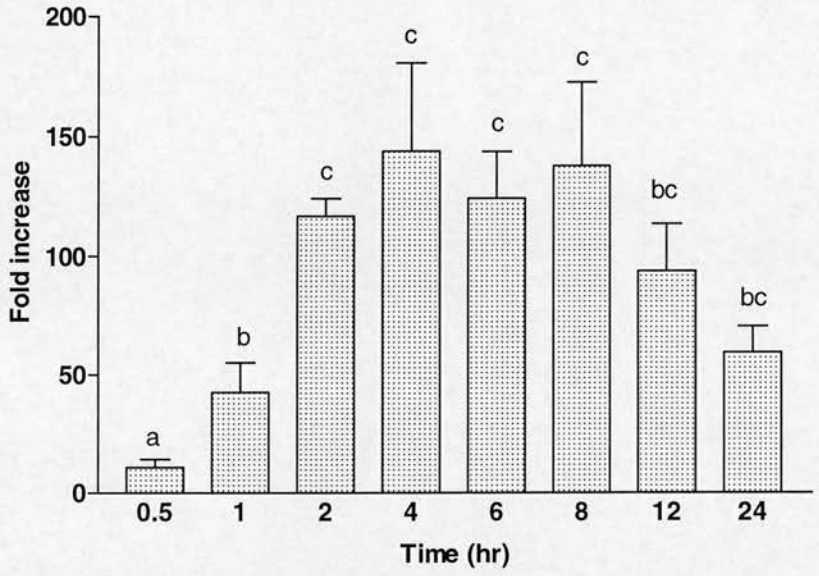

Fig. 4-4-2 Changes of cortisol output for high concentration ACTH time course. Cells were treated with or without ACTH (10^{-8} M) and the media were collected at various time points. Values from three independent experiments (two of them conducted in duplicate and one single treatment) are expressed as fold increase over the basal level (Mean ± SEM), n=5. Columns without the same superscript(s) letter were significantly different.

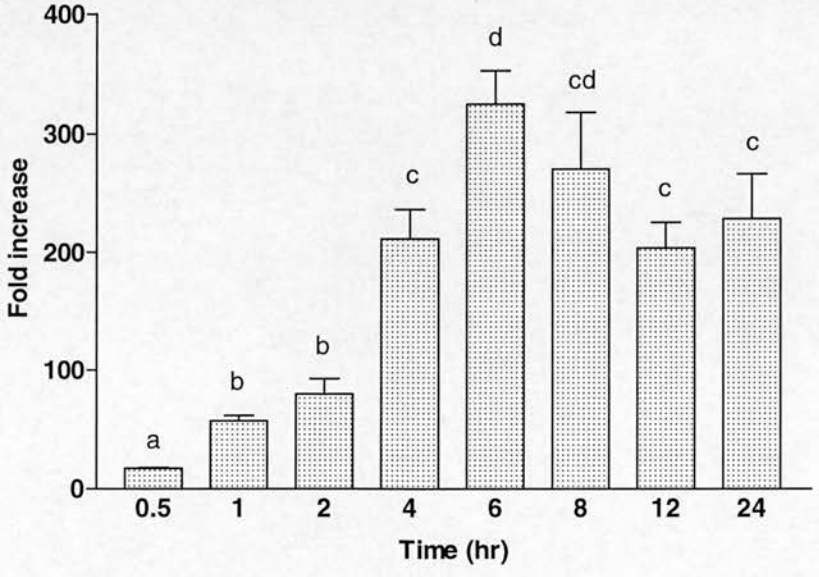

Fig. 4-4-3 Changes of cAMP levels for high-concentration ACTH time course. Cells were treated with ACTH (10^{-8} M) and the media were collected for cAMP assay at various time points. Values are expressed as fold increase over the corresponding basal level (mean ± SEM), n=3. Columns without the same superscript(s) were significantly different.

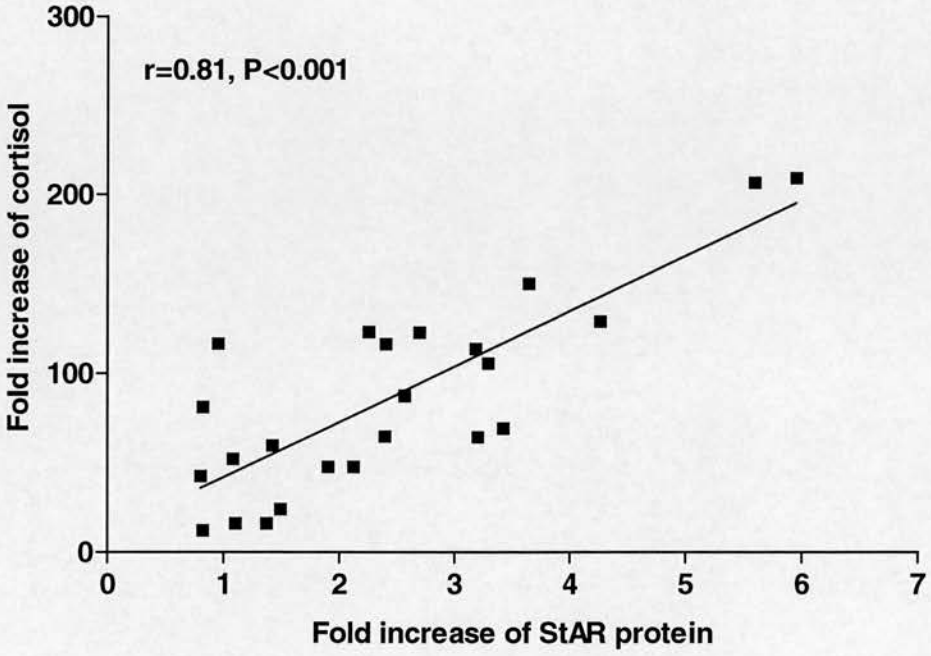

Fig. 4-4-4 Correlation between the changes of StAR protein and the cortisol levels for high-concentration ACTH time course.

Fig. 4-5-1a&b shows the levels of StAR protein in response to 10^{-12} M ACTH. The level at 6 hr was significantly different from those at 1, 2 and 12 ($P<0.01$), and 0.5, 4 and 24 hr ($P<0.05$).

Treatment with 10^{-12} M ACTH led to a maximal increase of cortisol at 2 hr and the cortisol level gradually declined thereafter (Fig. 4-5-2). The cortisol level at 2 hr was markedly different from the levels at 6, 8 and 12 ($P<0.05$), 0.5 hr ($P<0.01$) and 24 hr ($p<0.001$). Table 4-5 shows the details of the multiple comparisons of each level with others.

The changes of cAMP were extremely small across all time points; there was no notable difference among various time points (Fig. 4-5-3).

In contrast to high-concentration ACTH treatment, the changes of StAR protein were poorly correlated with cortisol levels ($r=0.04$, $P>0.05$; Fig 4-5-4).

Table 4-5 Multiple comparisons test for 10^{-12} M ACTH-induced cortisol levels

Comparison	P value
0.5 hr vs 1 hr	NS
0.5 hr vs 2 hr	P<0.01
0.5 hr vs 4 hr	NS
0.5 hr vs 6 hr	NS
0.5 hr vs 8 hr	NS
0.5 hr vs 12 hr	NS
0.5 hr vs 24 hr	NS
1 hr vs 2 hr	P<0.05
1 hr vs 4 hr	NS
1 hr vs 6 hr	NS
1 hr vs 8 hr	NS
1 hr vs 12 hr	NS
1 hr vs 24 hr	P<0.05
2 hr vs 4 hr	NS
2 hr vs 6 hr	P<0.05
2 hr vs 8 hr	P<0.05
2 hr vs 12 hr	P<0.001
2 hr vs 24 hr	P<0.001
4 hr vs 6 hr	NS
4 hr vs 8 hr	NS
4 hr vs 12 hr	P<0.05
4 hr vs 24 hr	P<0.01
6 hr vs 8 hr	NS
6 hr vs 12 hr	NS
6 hr vs 24 hr	P<0.05
8 hr vs 12 hr	NS
8 hr vs 24 hr	P<0.05
12 hr vs 24 hr	NS
NS: Not significant, i.e. P>0.05	

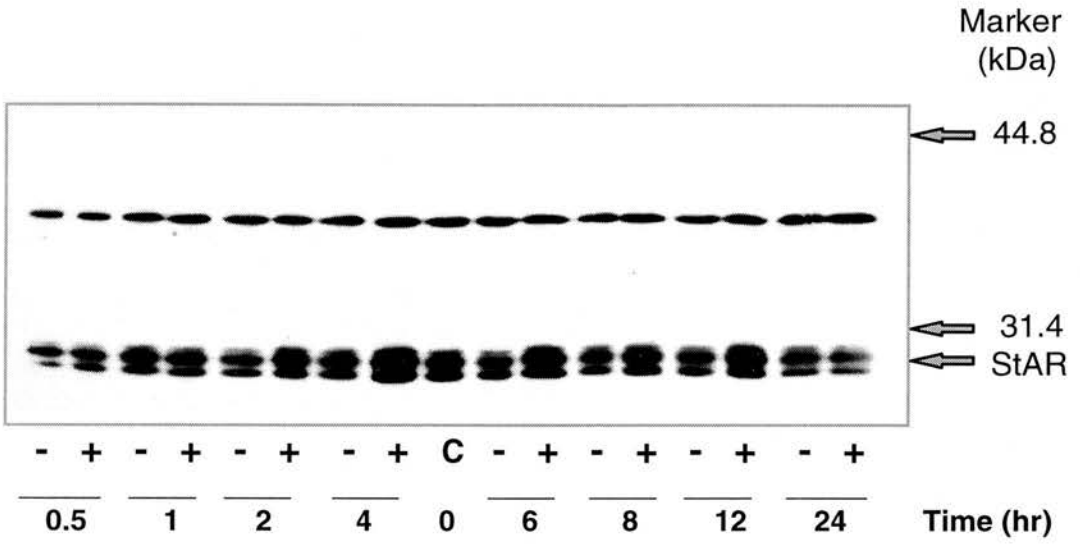

Fig. 4-5-1a Western immunoblot of StAR protein after low-concentration ACTH treatment. "-": Untreated cells; "+": ACTH (10^{-12} M) treated cells; C: Zero time point cells for control. All samples (25 μg protein) were resolved on a 12.5 % SDS-PAGE gel. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

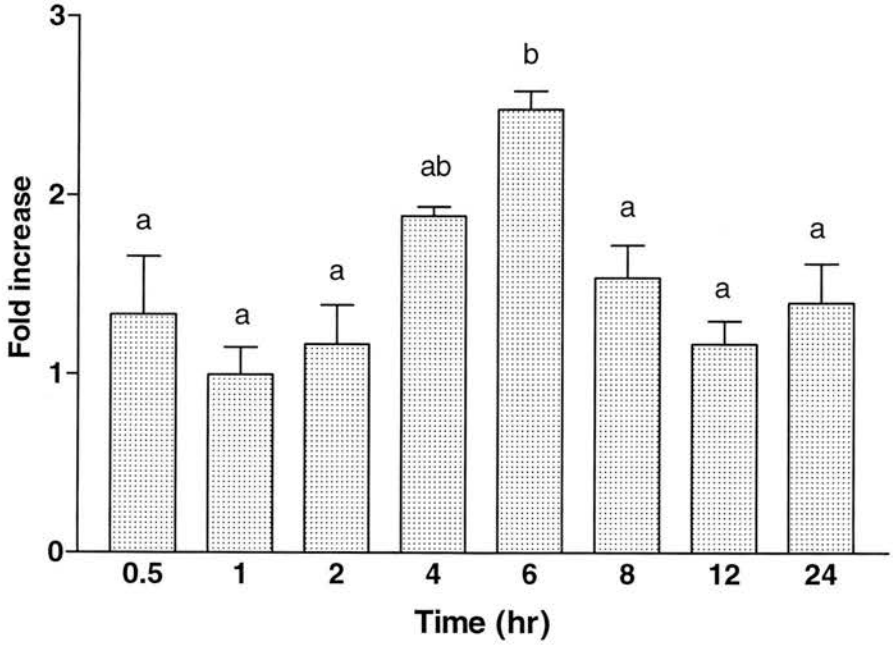

Fig. 4-5-1b Changes of StAR protein levels for low-concentration ACTH time course. Cells were treated with ACTH (10^{-12} M) and were harvested at various time points. Values from three independent experiments are expressed as fold increase over the corresponding basal level (mean ± SEM), n=3. Columns without the same superscript letter were significantly different.

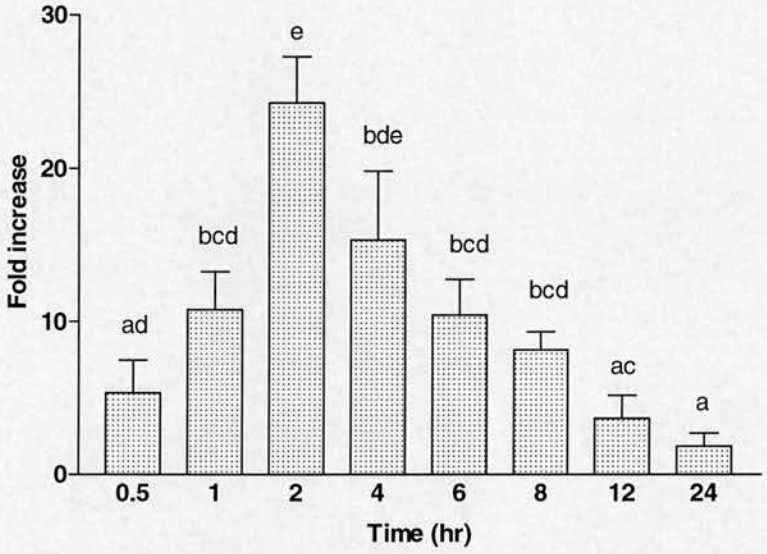

Fig. 4-5-2 Changes of cortisol levels for low-concentration ACTH time course. Cells were treated with ACTH (10^{-12} M) and the media were collected at various time points. Value from three independent are expressed as fold increase over the corresponding basal level (mean ± SEM), n=3. Columns without the same superscript letter were significantly different.

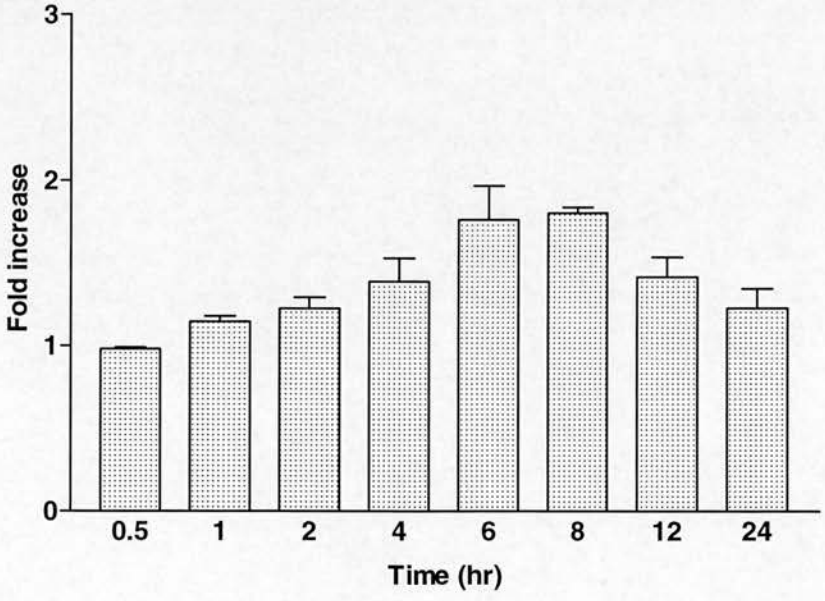

Fig. 4-5-3 Changes of cAMP levels of low-concentration ACTH time course. Cells were treated with or without ACTH (10^{-12} M) and the media were collected for cAMP assay at various time points. Values are expressed as fold increase over the corresponding basal levels (mean ± SEM), n=3. Data were tested by ANOVA, $P>0.05$.

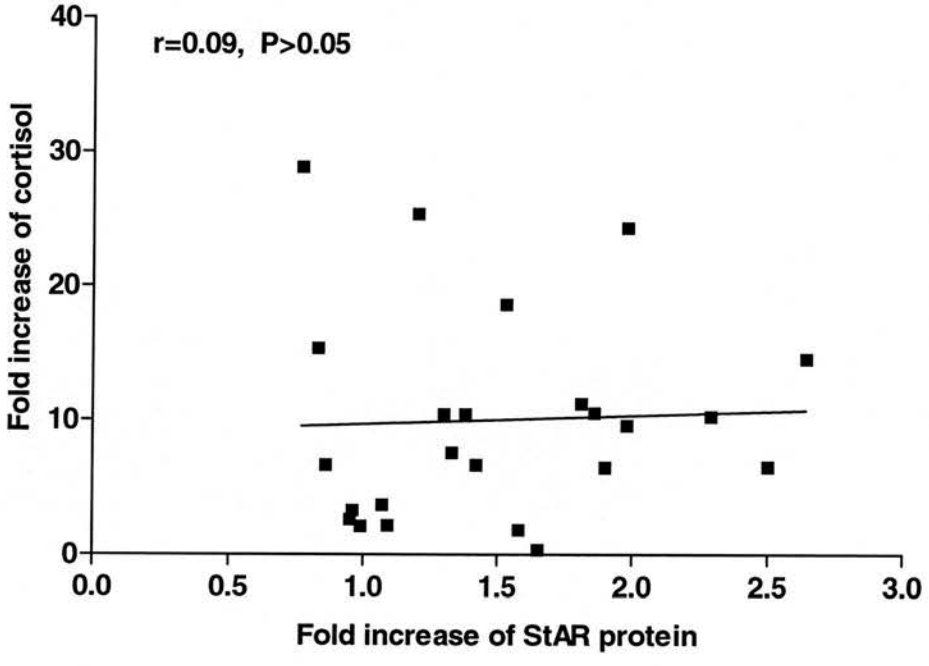

Fig. 4-5-4 Correlation between the changes of StAR protein and cortisol levels for low-concentration ACTH time course

4.6 EFFECTS OF ANG II AND THE COMBINED ANG II AND ACTH TREATMENT ON StAR PROTEIN AND CORTISOL SECRETION

The maximal StAR protein level occurred at 6 hr when cells were treated with Ang II (10^{-8} M) alone (Fig. 4-6-1a & b), and was different from the levels at 0.5, 4 and 24 hr ($P<0.05$), 2, 8 and 12 hr ($P<0.01$) and 1 hr ($P<0.001$). When cells were treated with both Ang II (10^{-8} M) and ACTH (10^{-12} M), the change of StAR protein at 6 hr was different from levels at 0.5, 1, 2, 12 and 24 hr ($P<0.001$), and also at 4 and 8 hr ($P<0.01$, Fig. 4-6-1a & c).

The cortisol changes in response to Ang II (10^{-8} M) were more modest compared to those of 10^{-12} M ACTH. The levels at 0.5 and 1 hr were different from those at 4, 6 and 8 hr ($P<0.05$, Fig. 4-6-2a).

In comparison with Ang II (10^{-8} M) or ACTH (10^{-12} M) alone, combined Ang II and ACTH resulted in further elevated cortisol production (32.5 ± 4.6 fold) at 2 hr, indicating that Ang II enhanced 10^{-12} M ACTH-induced cortisol secretion (24.3 ± 3.0 fold). The cortisol levels at 0.5 and 24 hr were different from those at 2, 4, 6 and 8 hr ($P<0.05$ for all tests) and there were no distinct changes compared with levels at 1 and 12 hr (Fig. 4-6-2b&c).

Correlation between the changes of StAR protein and cortisol induced by Ang II was not strong ($r=0.40$, $P<0.05$; Fig. 4-6-3a). The effect of the combined Ang II and ACTH (10^{-12} M) on StAR protein was not correlated with cortisol secretion ($r=0.35$, $P>0.05$; Fig 4-6-3b).

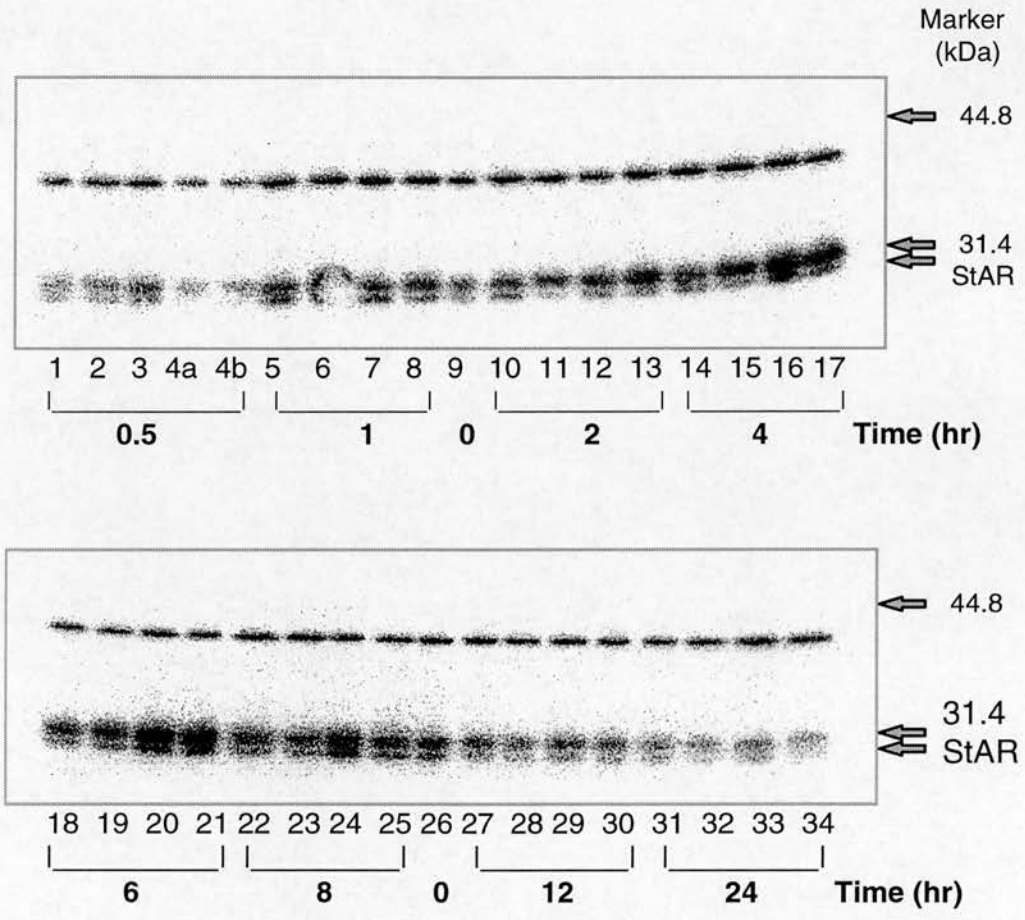

Fig. 4-6-1a Western immunoblots of StAR protein after Ang II and combined Ang II and low-concentration ACTH treatments (Bio-Rad image print). Lanes 1, 5 10, 14, 18, 22, 27 and 37: untreated cells; lanes 2, 6, 11, 15, 19, 23, 28, and 32: Ang II (10^{-8} M) treated cells; lanes 3, 7 12, 16, 20, 24, 29 and 33: ACTH (10^{-12} M) treated cells; lanes 4 (a & b), 8, 13, 17, 21, 25, 30 and 34. Ang II and ACTH treated cells; Lanes 9 and 26: Zero time point cells for control. All samples (25 μg protein) were resolved on 12.5 % SDS-PAGE gels. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:100,000) were diluted in PBS/10% Pierce blocking buffer containing 0.5% milk.

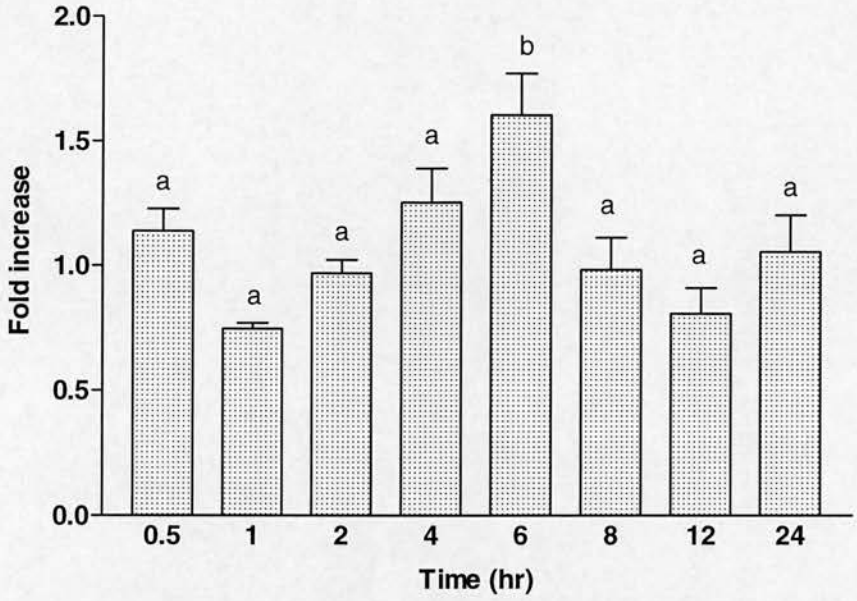

Fig. 4-6-1b Changes of StAR protein levels for Ang II treatment. Cells were treated with Ang II (10^{-8} M) and were harvested at various time points. Values from three independent experiments are expressed as fold increase over the corresponding basal level (mean ± SEM), n=3. Columns without the same superscript letter were significantly different.

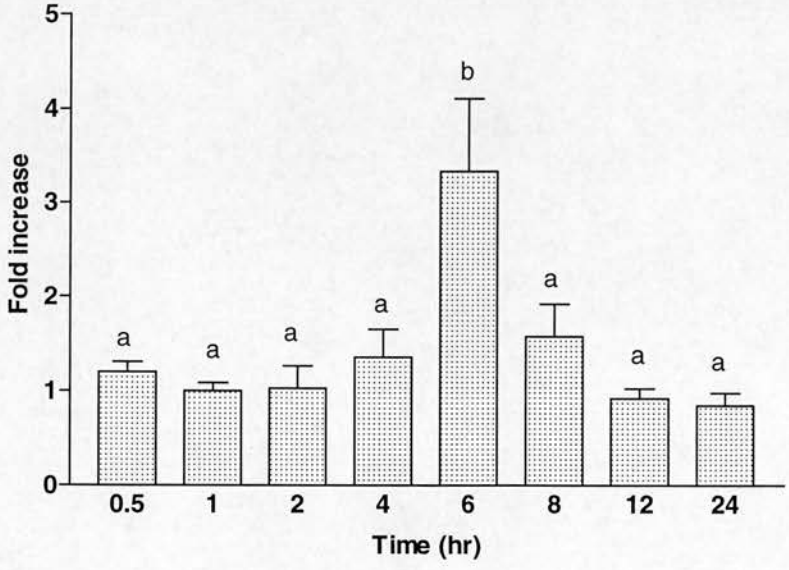

Fig. 4-6-1c Changes of StAR protein levels for the combined Ang II and low-concentration ACTH treatment. Cells were treated with Ang II (10^{-8} M) and ACTH (10^{-12} M) and were harvested at various time points. Values obtained from three independent experiments are expressed as fold increase over the corresponding basal level (mean ± SEM), n=3. Columns without the same superscript letter were significantly different.

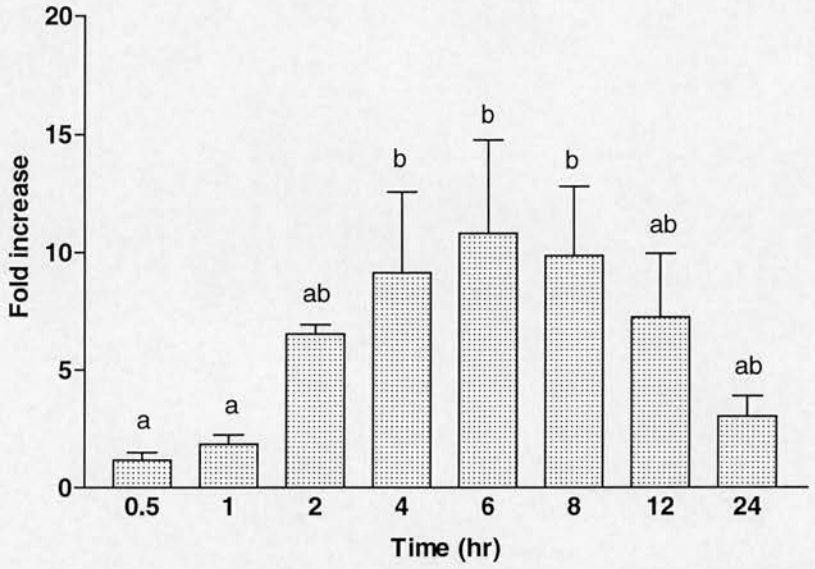

Fig. 4-6-2a Changes of cortisol levels for Ang II treatment. Cells were treated with Ang II (10^{-8} M) and ACTH (10^{-12} M), and the media were collected at various time points. Values obtained from three independent experiments are expressed as fold increase over the corresponding basal level (mean ± SEM), n=3. Columns without the same superscript letter were significantly different.

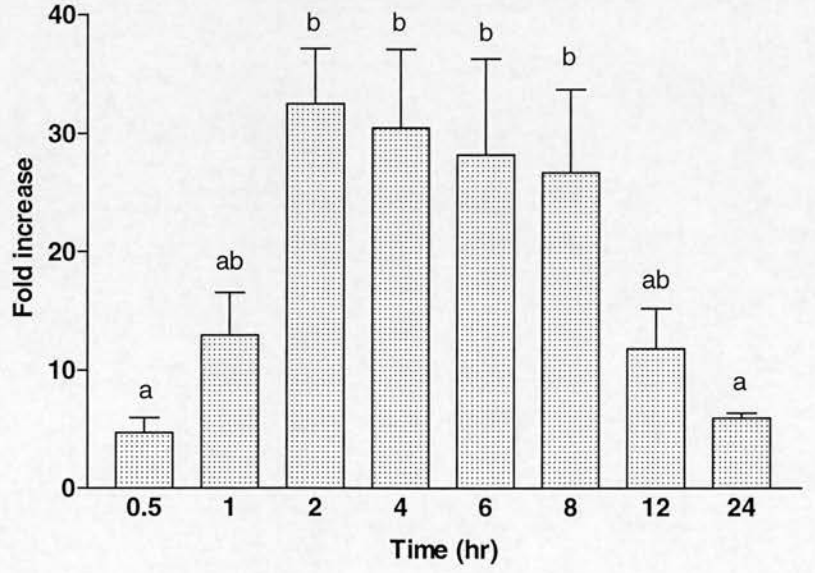

Fig. 4-6-2b Changes of cortisol levels for combined Ang II and low concentration ACTH treatment. Cells were treated with or without Ang II (10^{-8} M) and ACTH (10^{-12} M), and the media were collected at various time points. Values from three independent experiments are expressed as fold increases over the corresponding levels (mean ± SEM), n=3. Columns without the same superscript letter were significantly different.

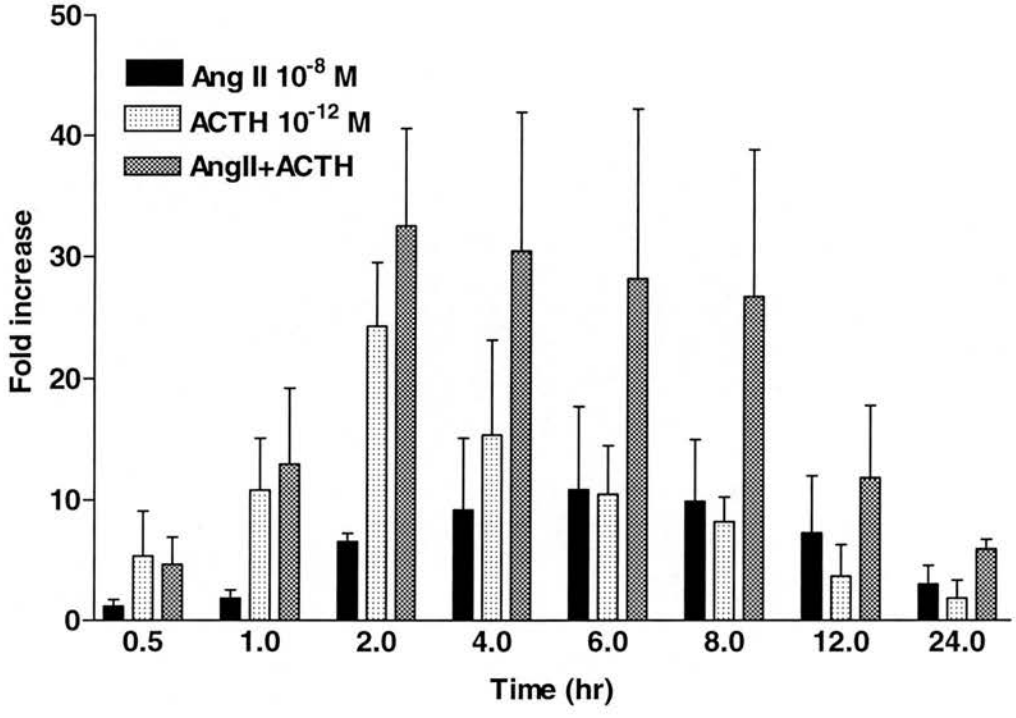

Fig. 4-6-2c Comparison of cortisol output induced by Ang II (10^{-8} M), ACTH (10^{-12} M) and the combined Ang II and ACTH.

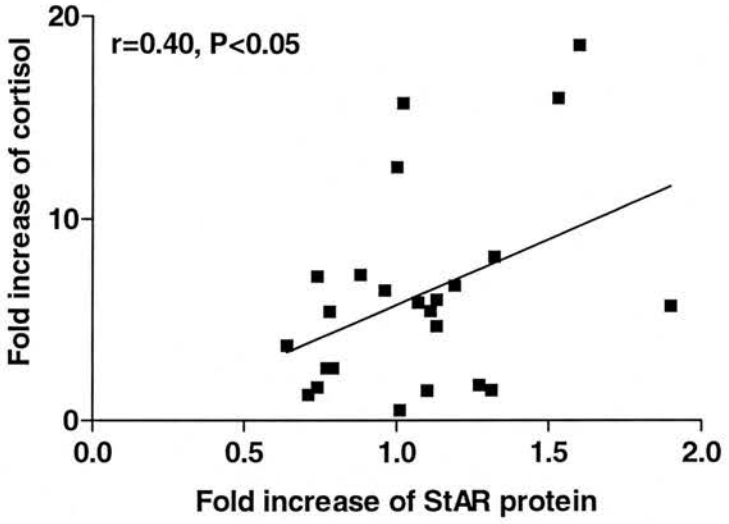

Fig. 4-6-3a Correlation between the changes in StAR protein and cortisol for Ang II (10^{-8} M) time course.

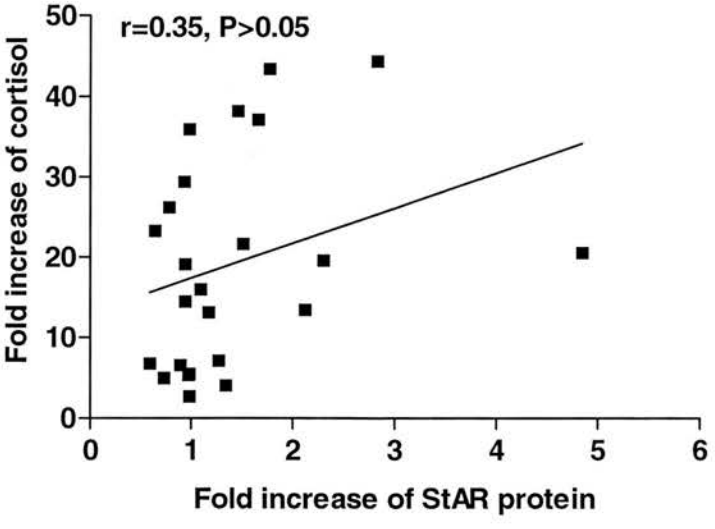

Fig. 4-6-3b Correlation between the changes in StAR protein and cortisol over combined Ang II (10^{-8} M) and ACTH (10^{-12} M) treatment.

4.7 DISCUSSION

The expression of StAR protein in adrenal ZF cells after ACTH treatment was monitored to provide a preliminary evaluation on 5 separate days of cell culture. On the basis of this work, a standard regime was established that initiated treatment on Day 3.

Data from other laboratories also support this selection. The binding of ACTH to cultured bovine ZF cells was lower on Day 1 than the level on Day 2. One suggested reason might relate to an incomplete recovery of cell membrane function after cell isolation by enzymatic digestion. From Day 2, the binding level remained fairly constant for at least further 2 days (Penhoat et al., 1989a). Furthermore, the daily changes of ACTH-R mRNA in freshly isolated and cultured bovine ZFR cells over five consecutive days demonstrated that the constitutive level of ACTH-R mRNA slightly decreased on Day 1 but increased thereafter. After investigating the time course of ACTH-R mRNA in the presence of ACTH (10^{-9} M), all their subsequent experiments were started on Day 2 or Day 3 and continued for one or two additional days (Penhoat et al., 1994).

Day 3 cells were also chosen for studies of steroidogenesis in other reports. For instance, experiments were usually started on Day 3 of culture when looking at Ang II receptor and steroidogenic responsiveness in bovine adrenal ZF and ZG cells (Penhoat et al., 1988). To study the effect of transforming growth factor β1 on StAR mRNA levels in a time course up to 24 hr, bovine adrenal ZF cells were used on Day 2 or 3 of primary culture (Brand et al., 1998).The 3-day cultured cells were also used for observing capacitative calcium entry in bovine adrenal ZF cells (Ebisawa et al., 2000).

In the current study the fundamental aspects of StAR protein expression, cortisol and cAMP formation in response to various concentrations of ACTH was investigated. The changes of StAR protein at 6 hr were similar to the results obtained by Yamazaki's group. In bovine adrenal ZFR cells StAR protein was increased by 6 hr incubation with ACTH (10^{-12} -10^{-8} M). Moreover, dose dependence of the expression of StAR protein by ACTH was correlated with that of

steroidogenesis (Yamazaki et al., 2000). The maximum cortisol and cAMP levels in response to ACTH were consistent with previous work in bovine adrenal ZF cells. A dose-response to ACTH (10^{-10}-10^{-6} M) demonstrated that the fluorogenic corticosteroid accumulation reached maximum at about 10^{-8} M ACTH during 60 min incubation (Peytremann et al., 1973). The ACTH (10^{-12}-10^{-8} M) dose-response curve for cAMP production showed that 10^{-9}-10^{-8} M ACTH induced the maximum cAMP levels. On the other hand, ACTH at 10^{-12} M resulted in elevated cortisol levels but no change in cAMP levels (Penhoat et al., 1989a).

In addition to exhibiting the general profiles of StAR protein and steroid production in response to ACTH treatment, the concentration response experiments have demonstrated different correlation between StAR protein and cortisol after 1 hr and 6 hr treatment. Following this work, the relationship between StAR protein and cortisol production was further evaluated throughout a 0.5-24 hr trophic hormone treatments.

With high-concentration ACTH (10^{-8} M) treatment, the maximum increase of StAR was 3.9 ± 1.1 fold at 4 hr and the elevated levels of StAR protein were sustained thereafter until 12 hr. A similar result showed that the expression of StAR protein was increased maximally at 6 hr by 10^{-7} M ACTH compared with the StAR levels at 2 and 18 hr (Nishikawa et al., 1996). The treatment with 10^{-8} M ACTH resulted in a considerable increase in steroid synthesis; cortisol showed a maximum 144.0 ± 36.6 fold increase, maintained at a high level from 2 until 12 hr. There was a strong positive correlation ($r=0.81$, $P<0.0001$) between StAR protein and cortisol production. StAR protein appeared tightly coupled to the rapid elevation of cortisol, indicating that the expression of StAR protein is crucial to steroidogenesis. This result is supported by a high correlation between StAR mRNA level and ACTH-induced cortisol secretion in bovine adrenocortical cells (Roy et al., 2000). However, after the low-concentration ACTH (10^{-12} M) treatment, the maximum increase of StAR was 2.5 ± 0.2 fold at 6 hr, with cortisol secretion increasing to 24.3 ± 4.3 fold at 2 hr. In fact, the cortisol secretion rose significantly prior to any changes in StAR protein synthesis, as confirmed by the lack of correlation ($r=0.04$, $P>0.05$) between the changes of StAR protein and cortisol levels. This finding is suggestive that the level of StAR protein may not be limiting for steroidogenesis at physiological levels of ACTH, but never the less a small "pool" of StAR might be critical to promote steroidogenesis.

Several mechanistic possibilities are plausible:

(i) The time lag between the expression of StAR protein and cortisol output implies that the acute response to ACTH may be independent of newly synthesised StAR protein. Low-concentration ACTH treatment clearly demonstrated distinct profiles between StAR protein and cortisol changes. Furthermore, with high-concentration ACTH treatment although there was strong correlation between StAR protein and cortisol changes overall, the acute response of steroid production was also ahead of marked increases in StAR protein.

The time lag of StAR protein expression has also been observed by other groups. An *in vivo* study showed that acutely ACTH elevated corticosterone to peak levels before any changes in total StAR protein in both intact and hypophysectomized (HPX) rat adrenals. Following *in vivo* ACTH stimulation the increase of StAR protein appeared to follow the rise in StAR mRNA in HPX rats and the ratio of the two measurements remained approximately constant during this process. Chronically (up to 24 hr) ACTH-treated HPX rats showed paralleled changes of adrenal StAR protein and serum cortocosterone levels (Ariyoshi et al., 1998). A correlation between StAR mRNA and protein as well as cholesterol levels demonstrated that maximum rates of cholesterol metabolism were attained in Y-1 cells within a few minutes of ACTH treatment, prior to any increase in StAR mRNA (Artemenko et al., 2000).

Acute ACTH treatment provoked a rapid increase in plasma corticosteroids in rat adrenal ZG and ZFR. An increase of StAR protein levels, after a delay, followed the increase of newly transcribed StAR mRNA (LeHoux et al., 1998). Moreover, some studies have shown that actinomycin D, a transcription inhibitor, did not change the hormonal stimulation of corticosterone production in rat adrenal (Farese, 1966), indicating that transcription may not be required for the acute response. Post-translational modifications of StAR precursor may occur during the early stimulatory phase and before the apparent translation of newly formed mRNA (LeHoux et al., 1998).

The somewhat slow translation step suggests that the mechanism for initiating the acute steroidogenesis may rely on a labile StAR pool or the modification of pre-

existing StAR protein or other factor(s). Acute steroidogenesis may therefore be independent of a net change of total StAR protein.

(ii) A subtle change in StAR protein may result in a profound response of steroid output. In other words, only a very small amount of StAR protein (e.g. at nanomolar level) is required for steroidogenesis, and this could be below the detection limits and therefore not measurable by semi-quantitative Western immunoblotting analysis. Arakane and co-workers have demonstrated that the purified C-His-tag StAR (lacking the first 62 amino acids) can increase rapidly pregnenolone production at concentrations as low as 20 nM within 20 min and that the stimulatory effect of C-His-tag StAR protein is dose- and time-dependent in isolated bovine corpus luteum mitochondria (Arakane et al., 1998). A possible mechanism may be that a very low level of high affinity StAR protein is functional for acutely elevating steroid levels, with further expression of StAR protein playing a key role on the sustenance of steroid synthesis.

It has been speculated by Strauss III's group that StAR protein can facilitate cholesterol transfer either through very high affinity stable interactions which are difficult to detect because of low number, or through a transient interaction which is too fast to be observed (Arakane et al, 1998). Conformational or topological changes of StAR protein might also be responsible for the regulation the cholesterol mobilisation.

(iii) Various StAR protein species classified in terms of isoelectric point value and molecular mass (i.e. pI vs. kDa) may also be important in regulation of steroidogenesis. The 2-D PAGE studies of *in vivo* expression of StAR protein demonstrated that the mobility of some StAR species varied between controls and Na^+-restricted rat adrenal ZG, suggesting that qualitative changes in StAR species content occurred in regulating aldosterone synthesis (Fleury et al., 2000). Using ^{35}S-methionine/cysteine pulse followed by cAMP stimulation, Artemenko and co-workers showed that two major StAR forms were diverted to two more acidic (phosphorylated) forms without a net increase in protein synthesis (Artemenko et al., 2000). The immunoblot results of whole adrenal gland mitochondria (MT) and supernatant (SN) revealed four bands in SN of

control rats and five bands in SN of ACTH-treated rats. The intensity of one band was increased and that of another one was decreased on treatment. Upon 2-D PAGE analysis, four StAR species were observed in untreated rat ZG and their intensity was increased after 5 hr, but not after 1 hr, *in vivo* ACTH treatment; additional acidic species also appeared (LeHoux et al., 1999).

iv) StAR protein is not the single limiting factor for cholesterol transfer but it may act in concert with other proteins such as PBR or an unidentified factor which may be an ancillary protein. Some evidence shows that adrenal PBR is able to change rapidly in response to acute ACTH treatment (Boujrad et al., 1994; Boujrad et al., 2000; Li and Papadopoulos, 1998; Papadopoulos et al., 1997). More recently, a study on fluorescence energy transfer has demonstrated that StAR protein and PBR are associated in the process of cholesterol delivery across the intramitochondrial membrane (West et al., 2001). StAR protein may act thus as part of a heteromeric complex StARosomes.

Moreover, StAR protein participates in a process involving GTP hydrolysis which is known to be important for delivering substrate to CYP11A (Kwoluru et al., 1995). Other factors also contribute to acute steroidogenesis. The proto-oncogene mRNA levels of *c-jun, c-fos, jun*-B and *fos*-B were increased within 30 min following ACTH administration in both rat adrenal ZG and ZFR. The high levels of these mRNA were only sustained for a short-term and diminished after 3 hr, and then returned to near or below basal levels at 5 hr (LeHoux et al., 1998). The rapid increase of *c-fos and jun*-B mRNA occurred after the addition of ACTH in bovine adrenal ZF cells (Viard et al., 1992). A comprehensive regulatory mechanism of StAR protein involvement in steroidogenesis may be through a dynamic combination of two or more of the above possibilities depending on the effective time and the agonist concentrations.

The relationships between StAR protein and cortisol secretion as well as cAMP formation at various concentrations of ACTH reflect not only that StAR protein and cAMP are important in steroidogenesis but also that other factor(s) and/or alternative pathways may be involved in steroidogenesis.

Low-dose ACTH is of clinical interest and can be useful to estimate the adrenal sensitivity under physiological conditions. The examination of the dose-response

relationship between plasma ACTH and cortisol concentrations after i.v. administration of increasing doses (1, 5 or 250 μg) of ACTH showed that an increase in plasma ACTH as low as 13 pM (i.e. the increase obtained with the 1 μg dose) induced a near maximal cortisol response (Darmon et al., 1999). 1 μg of ACTH administration via i.v. injection is the lowest ACTH dose to cause a maximal cortisol response and 0.6 μg of ACTH is sub-maximal dose for normal human (Dickstein et al., 1997). Concentration response curves and high- and low-concentration ACTH treatments indicated that the levels of cAMP were hardly changed at concentration of 10^{-12} M ACTH, while cortisol levels were significantly elevated. In other words, ACTH stimulates cortisol secretion at concentrations that are lower than those required to produced measurable in cAMP (Buckley and Ramachandran, 1981; Enyeart et al., 1996; Lefkowitz et al., 1971). In contrast, the cAMP levels were significantly increased when cells were treated with 10^{-11} M and higher concentrations of ACTH, paralleling with remarkable increases in cortisol levels. This is suggestive that cAMP may not be the sole second messenger which mediates the action of ACTH. The study of steroidogenesis in rat ZG cell demonstrated that ACTH activated a chloride channel via the Ras protein, providing evidence in which ACTH action in the adrenal is complex and most certainly not limited to the cAMP pathway, in particular for the low concentrations of ACTH. It is becoming increasingly apparent that ACTH triggers complex signalling systems in a closely interacting way (Gallo-Payet et al., 1999).

In addition to the mechanisms at the level of the second messenger systems, the promotion of steroidogenesis may also occur at a receptor level. The Scatchard analysis of bound and free ligand revealed two binding site populations for ^{125}I-ACTH in bovine adrenal ZF cells. One site has high affinity ($K_D = 2.3 \pm 0.4 \times 10^{-10}$ M) and low capacity (1910 ± 300 sites per cell), whereas another site is low affinity ($K_D = 1.6 \pm 0.6 \times 10^{-7}$ M) and high capacity (32,000 - 90,000 sites per cell) (Penhoat et al., 1989a). Possibly there are two types of receptors coupling with high- and low-concentration ACTH: one is able to bind both 10^{-8} M and 10^{-12} M ACTH, mediating cAMP and another signalling pathway respectively and one may principally be responsive to 10^{-8} M ACTH. Alternatively, it could be that one receptor class contains two different binding sites. The consequence of changes in receptor binding might lead to activation of different signalling pathways.

In mammals, ACTH and Ang II are the main hormones that regulate steroid production by the adrenal gland. Most work on the effects of Ang II are focused on the adrenal ZG cells. In addition, some evidence has shown that Ang II also regulates steroidogenesis in adrenal ZFR cells. Ang II can acutely stimulate cortisol production in bovine adrenal ZF cells (Crozat et al., 1986; Rainey et al., 1991).

The fold increase of cortisol production declined sharply after 2-4 hr treatment with low-concentration ACTH. However, it was sustained from 2 until 8 hr in the combined Ang II and ACTH treatment (Fig. 4-6-2b&c), suggesting a sensitising effect of Ang II on low-concentration ACTH induced-steroidogenesis. Both Ang II and ACTH induced the expression of StAR protein individually. However, the integrative effect of the combined Ang II and ACTH treatment on StAR protein did not correspond to additive cortisol output. The reason for this remains unclear.

Ang II and ACTH stimulate steroid secretion through their separate signalling systems. There may be at least two distinct mechanisms to link their effects on steroidogenesis. The results from combined 10^{-8} M Ang II and 10^{-12} M ACTH showed that Ang II enhanced ACTH-stimulated cortisol production, and suggests a steroidogenic regulatory process that may be important at physiological levels of ACTH.

The mechanism of the combined effects of ACTH and Ang II on steroidogenesis have been investigated by other groups. Interaction of ACTH ($2x10^{-7}$ M) and Ang II (10^{-6} M) for 3 min resulted in a cAMP increase greater than the sum of the effect of each hormone alone in bovine ZF cells (Peytremann et al., 1973). Ang II ($3x\ 10^{-8}$ M) treatment also potentiated the stimulatory actions of ACTH (10^{-9} M) on cAMP formation in the presence of extracellular calcium in COS-7 and bovine adrenal ZG cells (Baukal et al., 1994).

On the other hand, both ACTH and Ang II increased the mRNA levels of the ACTH receptor in a time- and dose-dependent manner. ACTH caused a 2.7-fold increase in 3.6 kb major transcript with an $ED_{50}=10^{-11}$ M and Ang II produced a 2.4-fold increase with an $ED_{50}=5x10^{-8}$ M. ACTH receptor number and mRNA are positively regulated by ACTH and Ang II, which regulates adrenocortical functions *in vivo*. The regulation of

ACTH receptors may be a mechanism by which ACTH and Ang II control steroidogenesis under both normal and pathological conditions (Lebrethon et al., 1994).

In addition, there exists a discrepancy with regard to the combined effect of Ang II and ACTH, that may be caused by different treatment regimes. For instance, after cells were pretreated with Ang II (10^{-7} M) for 48 hr and then treated with ACTH (10^{-9} M), Ang II inhibited the stimulatory effect of ACTH on its receptor (^{125}I-ACTH bound) by 50% in bovine adrenal ZF cells (Penhoat et al., 1989).

The combined Ang II and ACTH treatments might well induce a biphasic steroidogenic response. During acute and intermediate phases, Ang II may enhance ACTH-stimulated steroid synthesis. In contrast, chronically it may desensitise the effect of ACTH. One possible reason is that Ang II may inhibit the expression of one or more steroidogenic enzyme(s) after prolonged hormonal treatment. For example, Ang II appears to attenuate the ACTH-stimulated expression of CYP17 following a 24 hr treatment (Bird et al., 1993), resulting in a decrease in cortisol output.

Taken together, these results suggest a need to consider a more comprehensive notion to include parallel investigations on steroidogenesis, StAR protein and various intracellular signalling systems.

CHAPTER 5 SIGNALLING SYSTEMS AND THEIR RELATIONSHIPS TO StAR PROTEIN IN RESPONSE TO HIGH- AND LOW-CONCENTRATION ACTH TREATMENTS

5.1 INTRODUCTION

5.1.1. cAMP is not the sole second messenger of ACTH-induced steroidogenesis

It has been well established that cAMP mediates ACTH-induced steroidogenesis. This is based on two major pieces of evidence: ACTH stimulates a continuous increase in cAMP levels and the cAMP analogs such as Bu_2cAMP and 8-Br-cAMP are able to induce steroidogenesis to similar levels as ACTH treatment (Beall and Sayers, 1972; Cooke, 1999; Hanukoglu et al., 1990; Sala et al., 1979) .

Certain observations have weakened, however, an earlier hypothesis that cAMP is the sole second messenger for ACTH-induced steroidogenesis in the adrenal cortex. It has been found that there are concentrations of ACTH capable of stimulating steroidogenesis but apparently incapable of affecting cAMP production. cAMP appears to play a significant role as a second messenger for steroid synthesis only at ACTH concentrations greater than 10^{-11} M (Yamazaki et al., 1998). ACTH stimulates cortisol secretion at concentrations that may be lower than required to produce measurable changes in cAMP levels. Ca^{2+} was found to serve as a direct messenger in the physiological activation of steroidogenesis in isolated rat adrenocortical cells, whereas cAMP was claimed to be a subserving factor maintaining full steroidogenesis (Schulster and Schwyzer, 1980). cAMP and Ca^{2+} appear to act as dual second messengers in the ACTH-induced signalling pathways that are linked through I_{ac} K^+ channels. Both of these are required for a full steroidogenic response in adrenal ZF cells (Enyeart and Enyeart, 1998). Comparison of the effects of ACTH and NPS-ACTH (in which the position 2 on indole ring of tryptophan residue of ACTH was substituted by the *O*-nitrophenylsulfenyl group) on cAMP and steroid production were suggestive that although the integrity of the tryptophan residue in ACTH was essential for maximal cAMP formation, it was not so for maximal steroidogenesis.

This may also imply that another factor exists for mediating steroidogenesis (Moyle et al., 1973).

The results presented in Chapter 4 demonstrated that the significant increases in cortisol levels occurred at 10^{-12} M ACTH without notable changes of cAMP levels, suggesting the existence of other mechanism(s) for steroidogenesis in bovine adrenal ZF cells. Thus, further work was carried out to investigate a cAMP-independent signalling system, the arachidonic acid cascade, that may also mediate ACTH-induced steroidogenesis.

5.1.2 Arachidonic acid cascade and inhibitors

Briefly, the arachidonic acid (AA) cascade consists of two parts depending upon sequential activation process. The upstream pathways are the routes leading to AA liberation while the downstream pathways relate to the metabolism of AA to a variety of derivatives. Fig. 5-1-2 illustrates the AA cascades as well as action sites of the enzyme inhibitors .

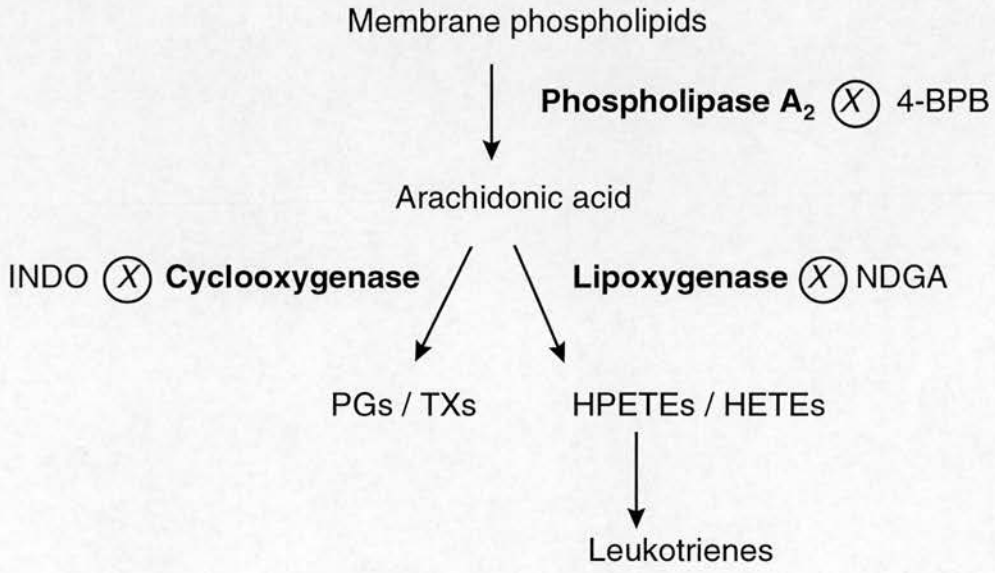

Fig. 5-1-2 Arachidonic acid metabolism and the inhibitors of pathways. 4-BPB: 4-Bromophenylacyl bromide; NDGA: Nordihydroguaiaretic acid; INDO: indomethacin; HPETE: Hydroperxyeisatetraenoic acid; HETE: Hydroxyeicosatetraenoic acid; PG: Prostaglandin; TX: Thromboxane; *X*: Inhibitory effect.

5.1.2.1 Inhibition of PLA_2 activity

PLA_2 catalyzes the hydrolysis of membrane phospholipids, releasing AA as the substrate or as a mediator for cellular process. As PLA_2 initiates the production of AA and a number of its metabolites that play roles as second messengers, it is obligate to determine whether the inhibition of PLA_2 affects steroidogenesis.

4-Bromophenacyl bromide (4-BPB, also known as p-BPB), a selective PLA_2 inhibitor, has been used in studying PLA_2 activity and steroidogenesis in rat cerebral cortex (Piomelli and Greengard, 1991), rat ZF cells (Mele et al., 1996) and bovine adrenal chromaffin cells (Morgan and Burgoyne, 1990). The roles of PLA_2 on cortisol production and StAR protein in bovine adrenal ZF cells remain unclear.

5.1.2.2 Downstream arachidonic acid cascade

Certain studies have demonstrated that the LOX and the COX aspects of the AA cascade functioned differentially in the adrenal cortex. In rat adrenal ZF cells pretreatment with INDO, a COX inhibitor, significantly suppressed prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production, indicating that AA can be metabolized to $PGF_{2\alpha}$ by COX. On the other hand, INDO had no effect on corticosterone levels, suggesting that PGs did not serve as mediators of adrenal steroidogenesis (Swartz et al., 1983). The results by Hirai et al also showed that INDO affected neither corticosterone production, nor cAMP levels. In contrast, two LOX products, 5-hydrooxyeicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4) were induced by ACTH, and furthermore augmented steroidogenesis in rat adrenocortical cells (Hirai et al., 1985). The results obtained by Yamazaki and co-workers indicated that LOX products might be involved steroidogenesis. In bovine ZF cells, the addition of AA or 15-hydroperoxyeicosatetraenoic acid (15-HPETE) increased pregnenolone production and the stimulatory effect of AA was blocked by NDGA (100 μM), a selective LOX inhibitor and AA861 (50 μM), a specific 5-LOX inhibitor (Yamazaki et al., 1996).

5.1.3 *Aims and experimental strategies*

A comprehensive mechanism by which ACTH stimulates steroidogenesis remains incompletely delineated. This work aimed at investigating the regulatory mechanisms of ACTH-induced steroidogenesis, particularly by identifying the signalling pathways operative at physiologically relevant ACTH levels. The experiments were conducted using a physiological concentration of ACTH (10^{-12} M) in parallel with a supra-physiological concentration of ACTH (10^{-8} M). Various compounds were used to examine the effects of AA and its metabolites on ACTH-induced steroidogenesis and StAR protein (Fig. 5-1-3a). Based on results presented in Chapter 4, the 1 hr time point was selected for examining the acute effects of ACTH on steroid production, while a 6 hr treatment was selected for observing the expression of StAR protein.

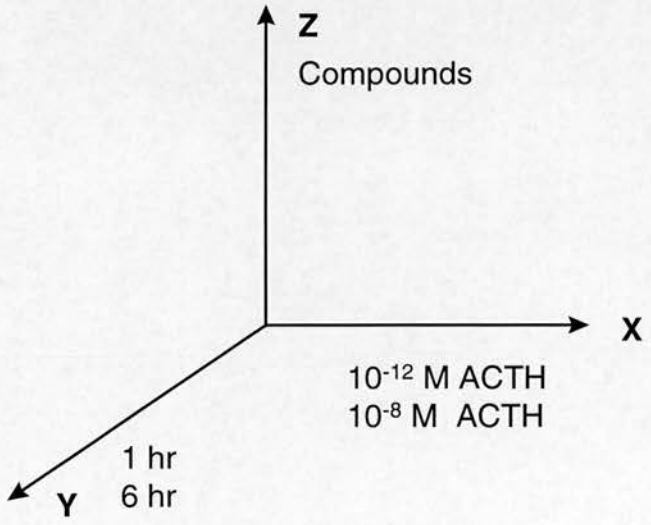

Fig. 5-1-3a Strategy of investigating the second messenger system of ACTH-stimulated steroidogenesis. X axis: Cells were treated with 10^{-12} M or 10^{-8} M ACTH; Z axis: Compounds including 4-BPB, NDGA, INDO and AA; Y axis: Cells and media were collected at 1 and 6 hr after treatments.

The aims of this work illustrated in Fig. 5-1-2 and Fig. 5-1-3b were:

- To examine whether the release of AA by PLA_2 affects steroidogenesis.
- To test which downstream branch(es) of the AA cascade is/are involved in steroidogenesis.
- To evaluate the correlation between the concentrations of ACTH and signalling pathways.
- To evaluate the correlation between the activation of the AA pathways and StAR protein levels.
- To examine whether there is an interaction between the AA cascade and the cAMP pathway.
- To test the specificity of compounds using 27-nor-5-cholesten-3β-ol-25-one, a readily diffusible substrate for steroidogenesis.
- To compare the effects of regular BSA and fatty acid free BSA on cortisol production.

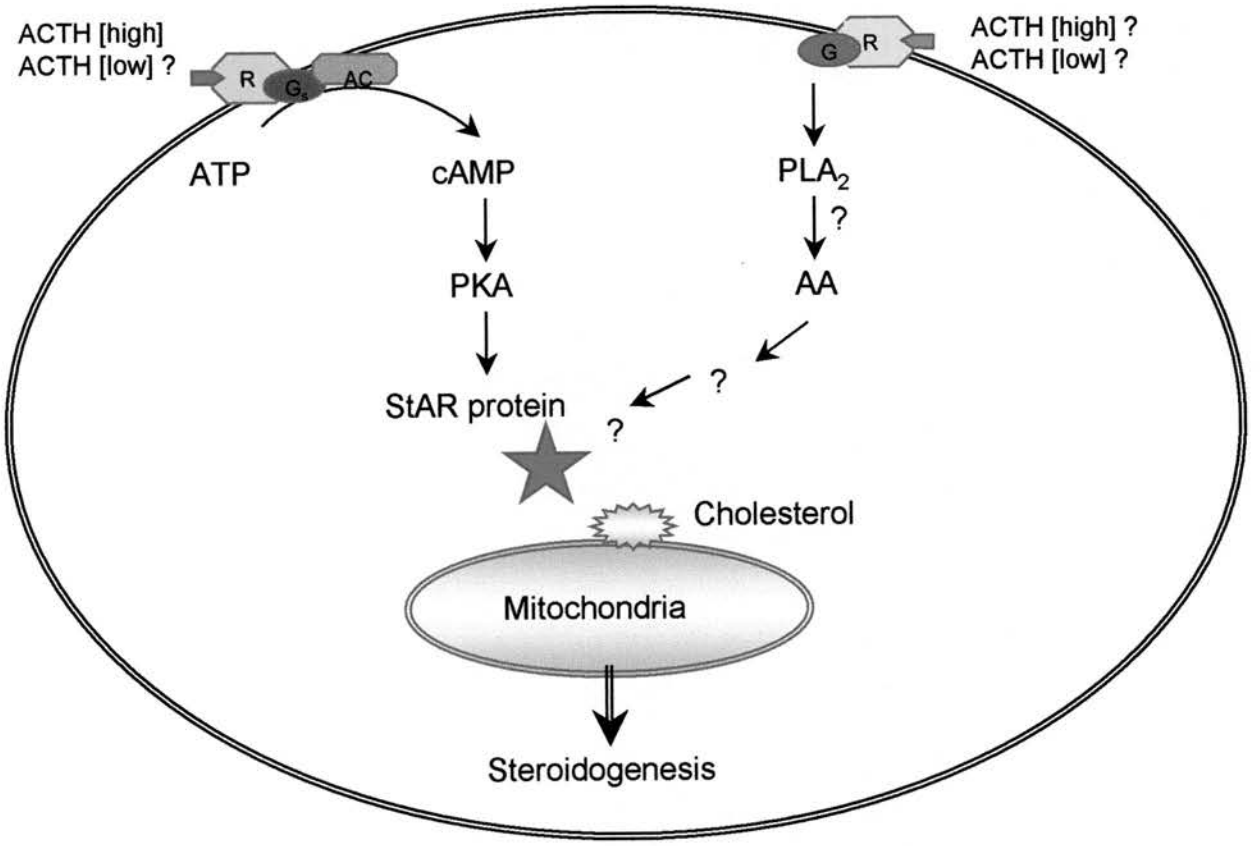

Fig. 5-1-3b Signalling pathways of steroidogenesis in response to ACTH in adrenocortical cells. R: Receptor; G: G-protein; AC: Adenylate cyclase; PLA_2: Phospholipase A_2; AA: Arachidonic acid; PKA: Protein kinase A. ACTH[high]: High concentrations of ACTH; ACTH[low]: Low concentrations of ACTH.

5.2 ROLE OF PHOSPHOLIPASE A_2

5.2.1 Effect of 4-BPB on cortisol output

At 1hr, the levels of cortisol after ACTH (10^{-12} M) treatment were markedly decreased by 4-BPB at concentrations ranging from 10^{-7}-10^{-5} M ($P<0.05$), whereas, the levels of cortisol stimulated by ACTH (10^{-12} M) were not affected by 4-BPB at 6 hr (Fig. 5-2-1A-upper panel). 4-BPB (10^{-5} M) only slightly inhibited ACTH (10^{-8} M)-induced cortisol output at 1 hr. All concentrations of 4-BPB failed to alter the increases in cortisol by ACTH (10^{-8} M) at 6 hr (Fig. 5-2-1A-lower panel).

In the medium containing 0.2% fatty-acid-free (FAF) BSA, 4-BPB significantly inhibited ACTH (10^{-12} M)-induced cortisol production at concentrations of 10^{-6} and 10^{-5} M ($P<0.05$). The effect of 4-BPB on cortisol output was not significant at 6 hr (Fig. 5-2-1B-upper panel). 4-BPB (10^{-7}-10^{-5} M) markedly impaired ACTH (10^{-8} M)-induced cortisol increase ($P<0.01$) at 1 hr, but not that at 6 hr (Fig. 5-2-1B-lower panel).

In summary, the effects of 4-BPB on cortisol occurred acutely (1 hr) and the inhibitory effect on 10^{-8} M ACTH-induced cortisol was more clearly seen in the medium containing 0.2% FAF BSA than that in medium containing 0.2% BSA.

5.2.2 Effect of 4-BPB on StAR protein levels

Western blotting failed to reveal any change in total StAR protein induced by ACTH (10^{-12} M) in the presence of various concentrations of 4-BPB compared with ACTH alone at both 1 and 6 hr treatment periods (Fig. 5-2-2a-upper & lower panels; Fig. 5-2-2b-upper panel).

Similarly, 4-BPB had no effect on ACTH (10^{-8} M)-stimulated total StAR protein (Fig 5-2-2a-upper & lower panels; Fig. 5-2-2b-lower panel).

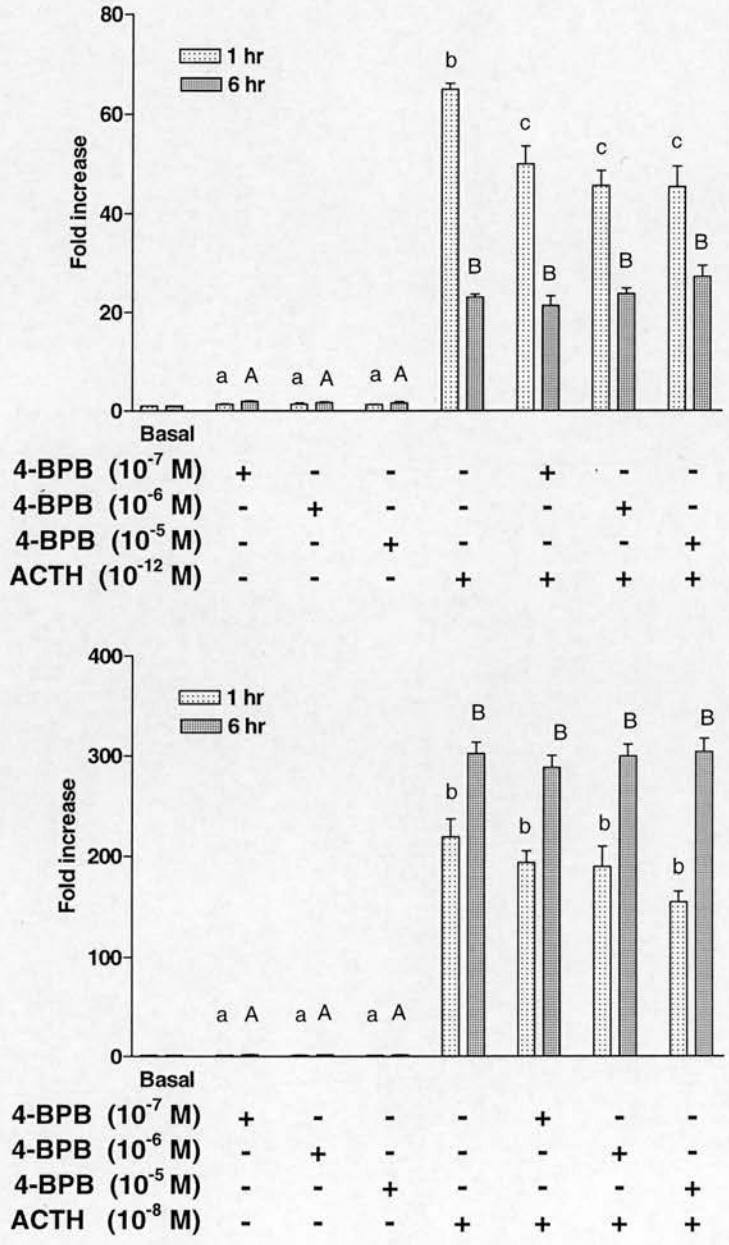

Fig. 5-2-1A Effects of 4-BPB on cortisol levels induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments. Cells were pretreated with various concentrations of 4-BPB for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time point data and letters in upper case indicate 6 hr time point data.

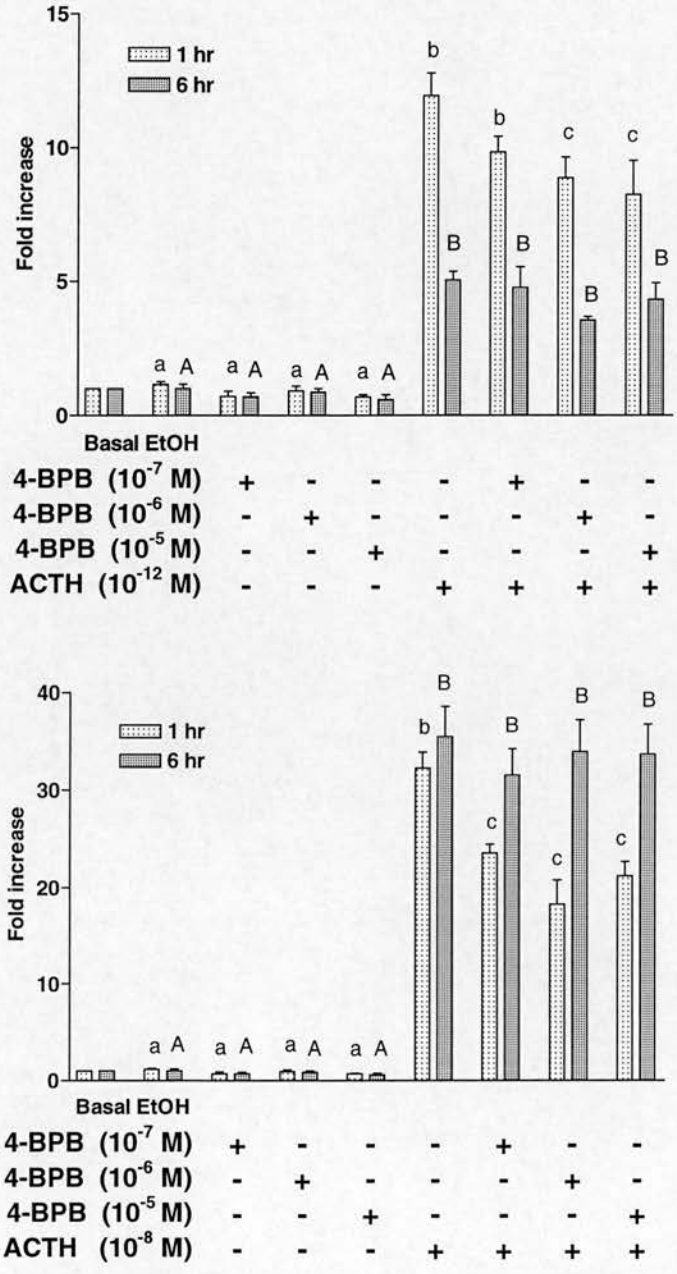

Fig. 5-2-1B Effect of 4-BPB on cortisol levels induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments in FAF BSA medium. Cells were pretreated with various concentrations of 4-BPB for 15 min prior to the addition of ACTH (10^{-12} or 10^{-8} M). Values were expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript(s) are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

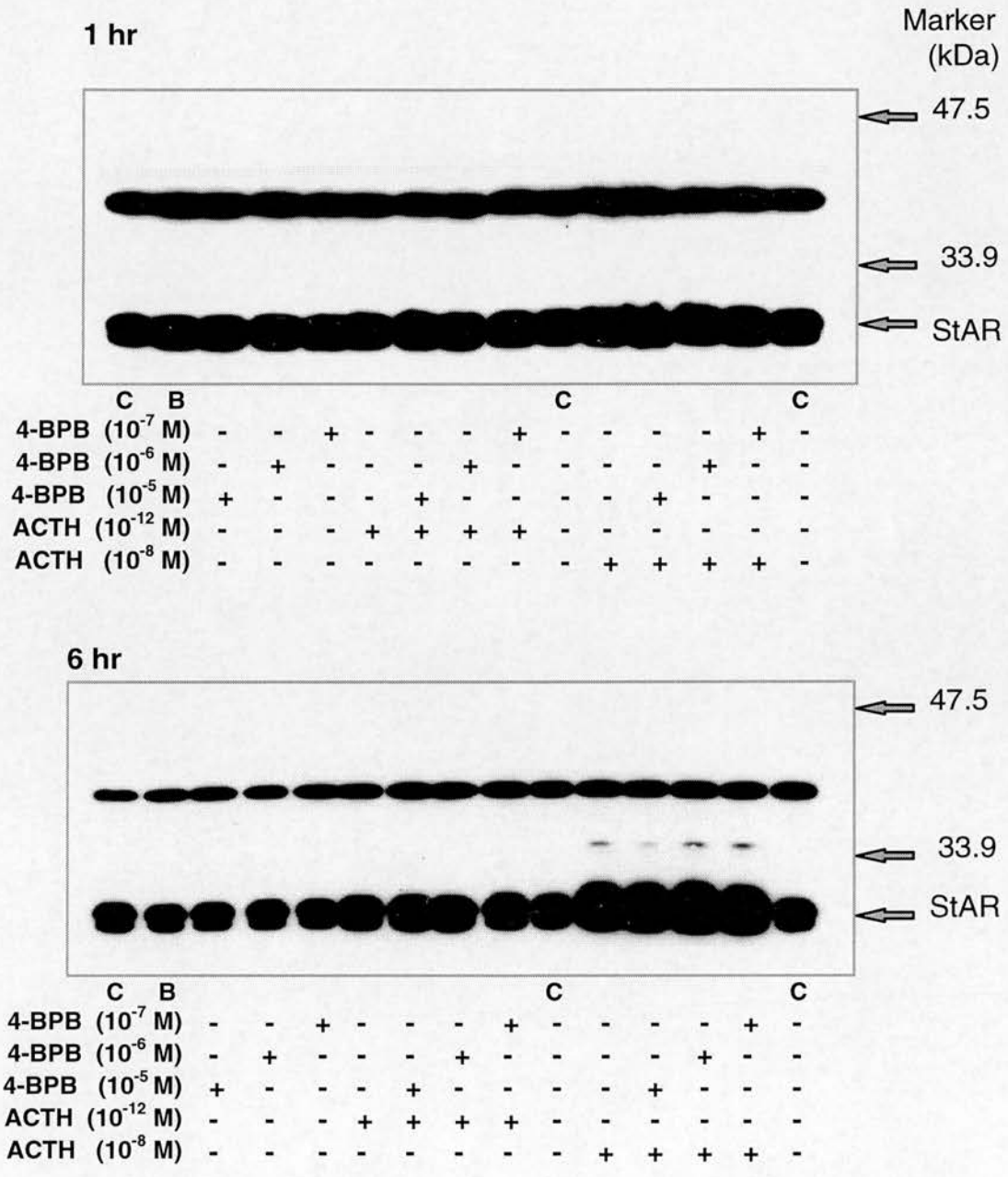

Fig. 5-2-2a Western immunoblots of StAR protein in response to various concentrations of 4-BPB at 1 hr (upper panel) and at 6 hr (lower panel). C: Zero time point untreated cells. B: Basal (untreated cells at 1 or 6 hr). Samples (25 µg protein) were resolved on 12.5% large SDS-PAGE gels and blotted on to PVDF membranes. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

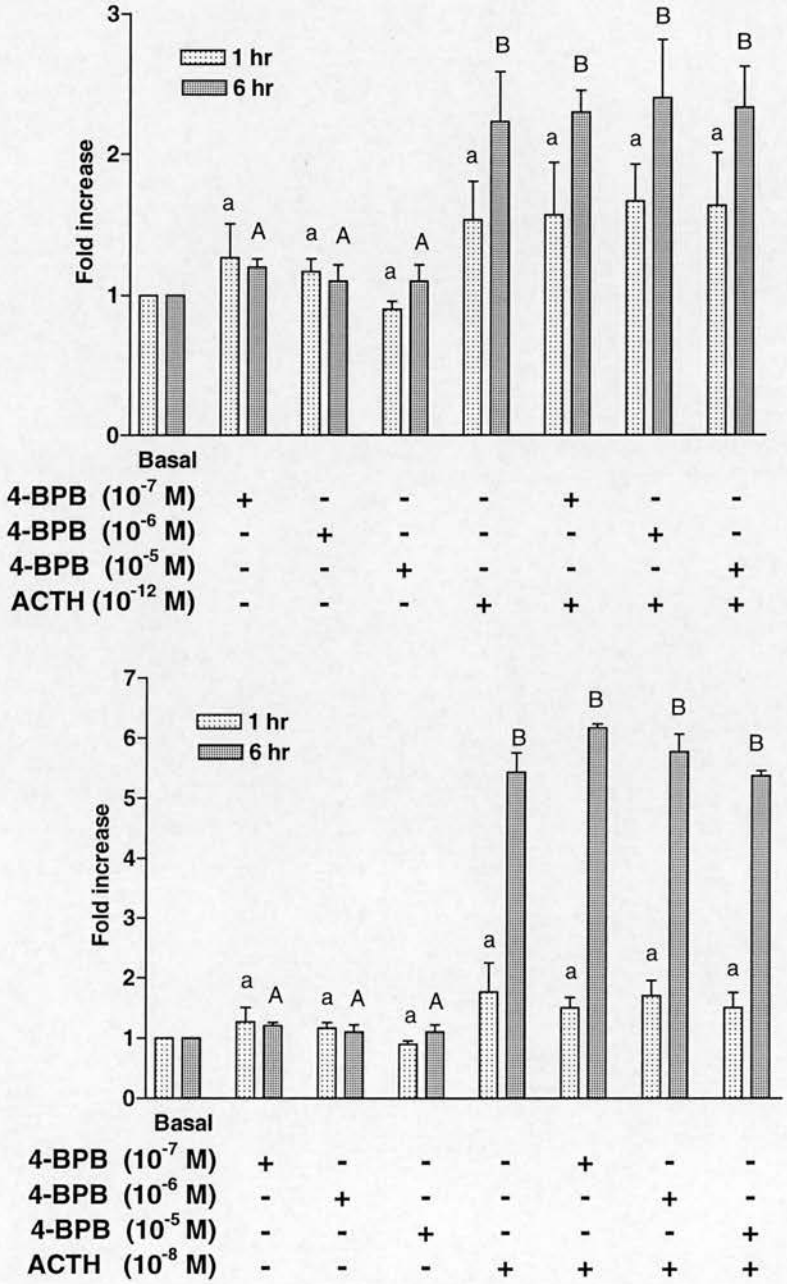

Fig. 5-2-2b Effect of 4-BPB on StAR protein induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments. Cells were pretreated with various concentrations of 4-BPB for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels in a representative experiment performed in triplicate (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

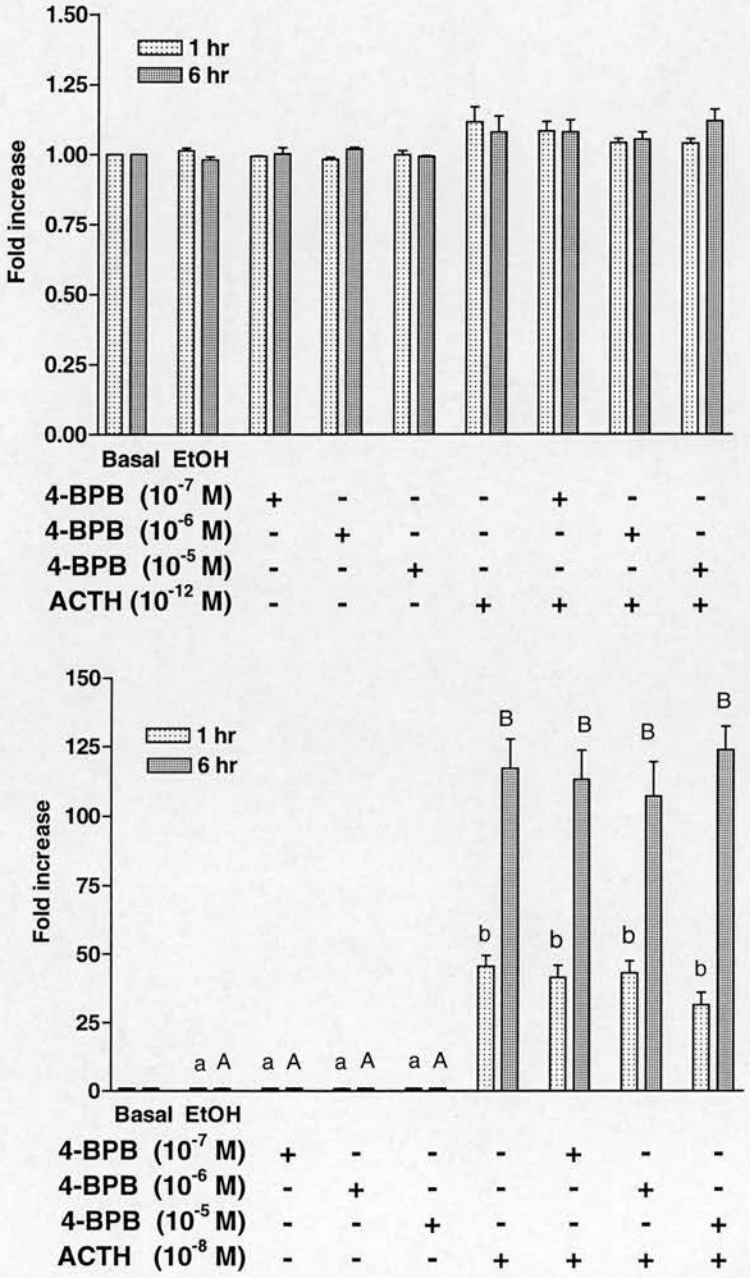

Fig. 5-2-3 Effect of 4-BPB on cAMP levels induced by low-concentration ACTH (upper panel) high-concentration ACTH (lower panel) treatments. Cells were pretreated with various concentrations of 4-BPB for 15 min prior to the addition of ACTH (10^{-12} or 10^{-8} M). Values are expressed as fold increase over basal levels in a representative experiment performed in triplicate (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

5.2.3 Effect of 4-PBP on cAMP formation

ACTH (10^{-12} M) did not alter cAMP levels and the addition of 4-BPB similarly had no effect at 1 either or 6 hr treatment period (Fig 5-2-3-upper panel). A high concentration of 4-BPB (10^{-5} M) caused a minor decrease in ACTH (10^{-8} M)-induced cAMP level at 1 hr of treatment but not at 6 hr period (Fig. 5-2-3-lower panel).

5.3 ROLE OF THE LIPOXYGENASE PATHWAY

5.3.1 Effect of NDGA on cortisol output

Fig. 5-3-1A-upper panel showed that NDGA significantly reduced ACTH (10^{-12} M)-induced cortisol increases ($P<0.05$ at $2x10^{-6}$ M and 10^{-5} M; $P<0.001$ at $5x10^{-5}$ M) and the inhibitory effects at $5x10^{-5}$ M NDGA was more marked than those at $2x10^{-6}$ and 10^{-5} M NDGA ($P<0.01$) after 1 hr. The stimulatory effect of ACTH (10^{-12} M) was reduced in the presence of $5x10^{-5}$ M NDGA ($P<0.01$) after 6 hr treatment.

As shown in Fig. 5-3-1A-lower panel, $5x10^{-5}$ M NDGA significantly inhibited ACTH (10^{-8} M)-stimulated cortisol increase after 1 hr ($P<0.01$) and 6 hr periods($P<0.001$).

With medium containing 0.2% FAF BSA, NDGA notably inhibited the cortisol levels induced by 10^{-12} M ACTH ($P<0.01$ at 10^{-5}; $P<0.001$ at $3x10^{-5}$ and $5x10^{-5}$ M). Moreover, the inhibitory effects were concentration-dependent. Compared to ACTH (10^{-12} M) treatment alone, the cortisol level in the presence of $3x10^{-5}$ M NDGA was lower than that at 10^{-5} M ($P<0.01$) and the level at $5x10^{-5}$ M was lower than that at 10^{-5} M ($P<0.001$) and $3x10^{-5}$ M ($P<0.05$) after 1 hr of treatment (Fig. 5-3-1B-upper panel).

NDGA inhibited ACTH (10^{-8} M)-induced cortisol increases ($P<0.01$ at $3x10^{-5}$ M and $P<0.001$ at $5x10^{-5}$ M respectively) at 1 hr. Similarly, NDGA also inhibited ACTH (10^{-8} M)-induced cortisol increases ($P<0.05$ at $3x10^{-5}$ M $P<0.01$ at $5x10^{-5}$ M respectively) at 6 hr. The effect of NDGA at $5x10^{-5}$ M on cortisol output was more marked than that at $3x10^{-5}$ M ($P<0.05$; Fig. 5-3-1B-lower panel).

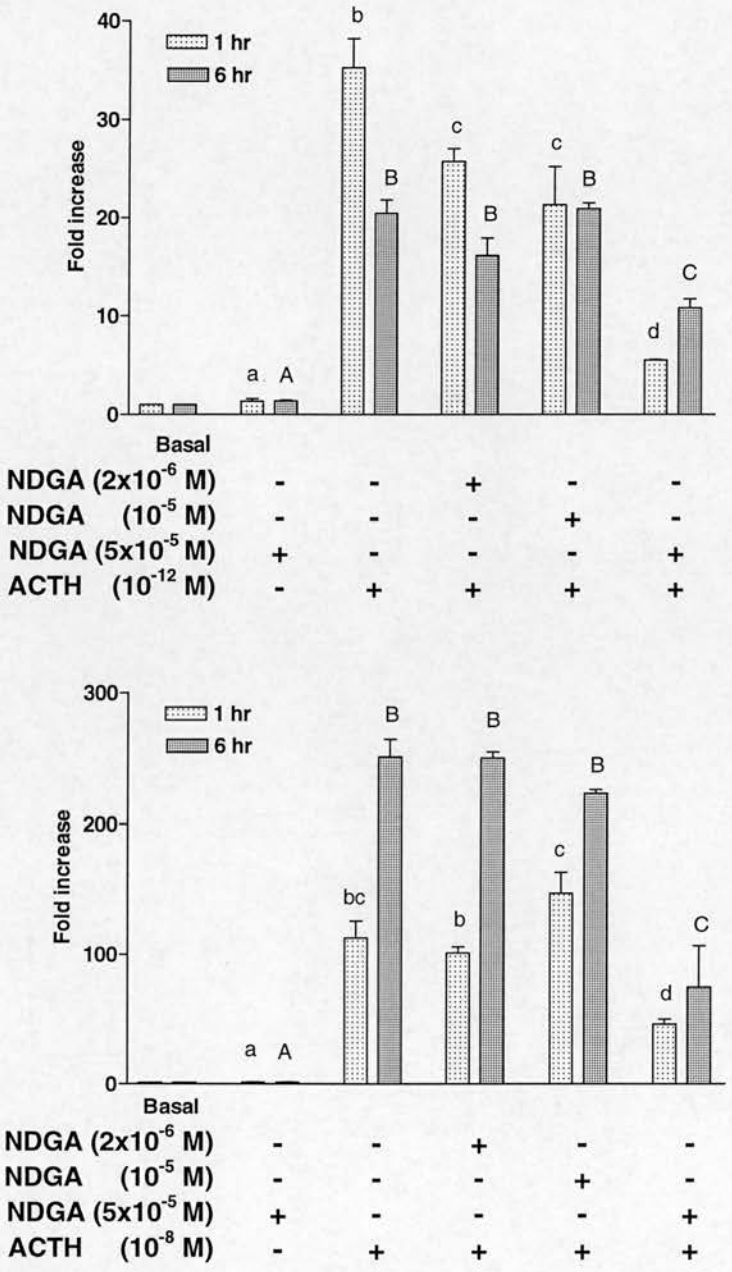

Fig. 5-3-1A Effect of NDGA on cortisol levels induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments. Cells were pretreated with various concentrations of NDGA for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

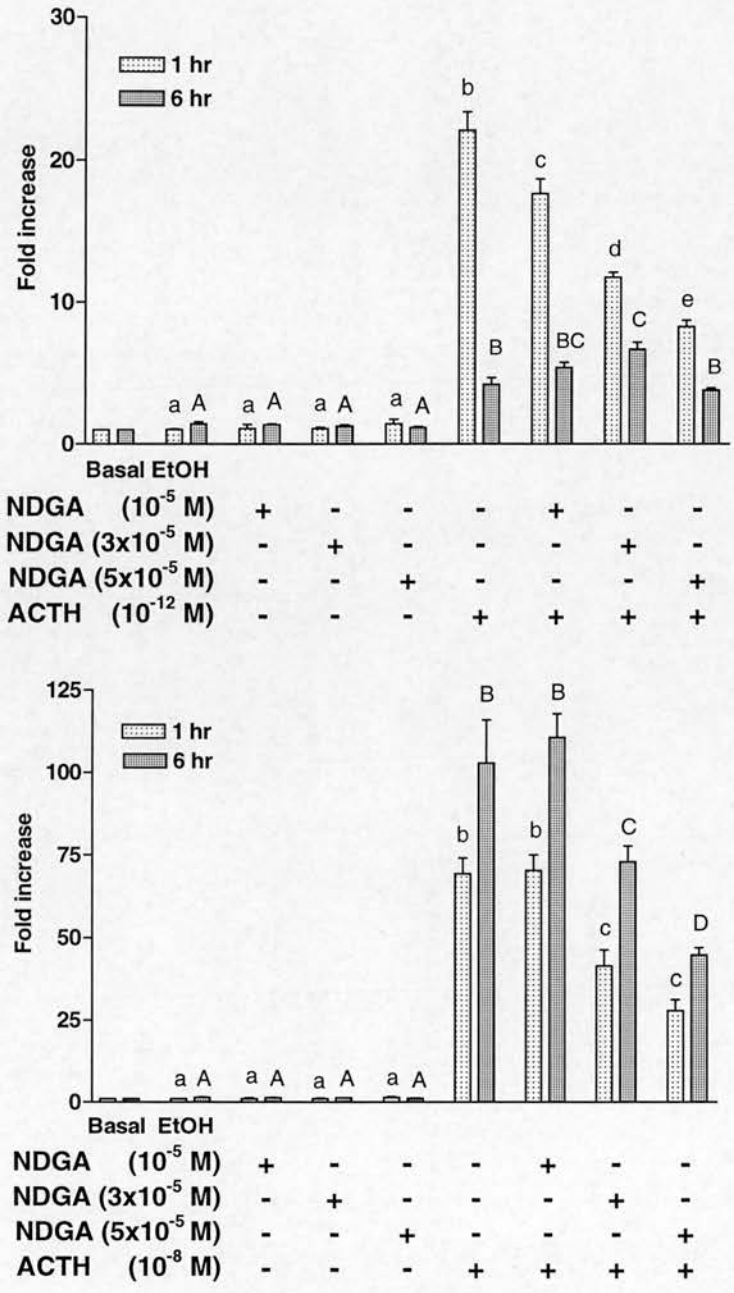

Fig. 5-3-1B Effect of NDGA on cortisol levels induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments in FAF BSA medium. Cells were pretreated with various concentrations of NDGA for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

5.3.2 Effect of NDGA on StAR protein level

There was no marked difference between the levels of StAR protein induced by ACTH (10^{-12} M) and those in the presence of ACTH as well as various concentrations of NDGA (Fig. 5-4-2a-upper and lower panels; Fig. 5-3-2b-upper panel).

NDGA at $2x10^{-6}$ and 10^{-5} M appeared to slightly affect ACTH (10^{-8} M)-induced StAR protein levels (Fig. 5-4-2a-upper and lower panels; Fig. 5-3-2b-lower panel).

5.3.3 Effect of NDGA on cAMP formation

There was no changes by NDGA alone and in the presence of 10^{-12} M ACTH on cAMP levels after 1 and 6 hr treatments (Fig. 4-3-3-upper panel). At 1 hr, NDGA inhibited ACTH (10^{-8} M)-stimulated cAMP production ($P<0.05$ at 10^{-6} M; $P<0.01$ at $5x10^{-5}$ M). The cAMP level in the presence of $5x10^{-5}$ M NDGA was different from that at $2x10^{-6}$ M ($P<0.05$). Only $5x10^{-5}$ M NDGA significantly reduced 10^{-8} M ACTH-induced cAMP increases at 6 hr ($P<0.05$, Fig. 5-3-3-lower panel).

5.4 ROLE OF THE CYCLOOXYGENASE PATHWAY

To examine whether the COX pathway of the AA cascade plays any role in ACTH action, the effects of INDO on cortisol output, StAR protein and cAMP were investigated.

5.4.1 Effect of INDO on cortisol output

INDO at 10^{-5} and 10^{-4} M potentiated ACTH (10^{-12} M)-induced increases in cortisol ($P<0.05$) at 1 hr but had no significant effect at 6 hr (Fig. 5-4-1A-upper panel). INDO did not affect ACTH (10^{-8} M)-induced cortisol increases either 1 or 6 hr (Fig. 5-4-1A-lower panel).

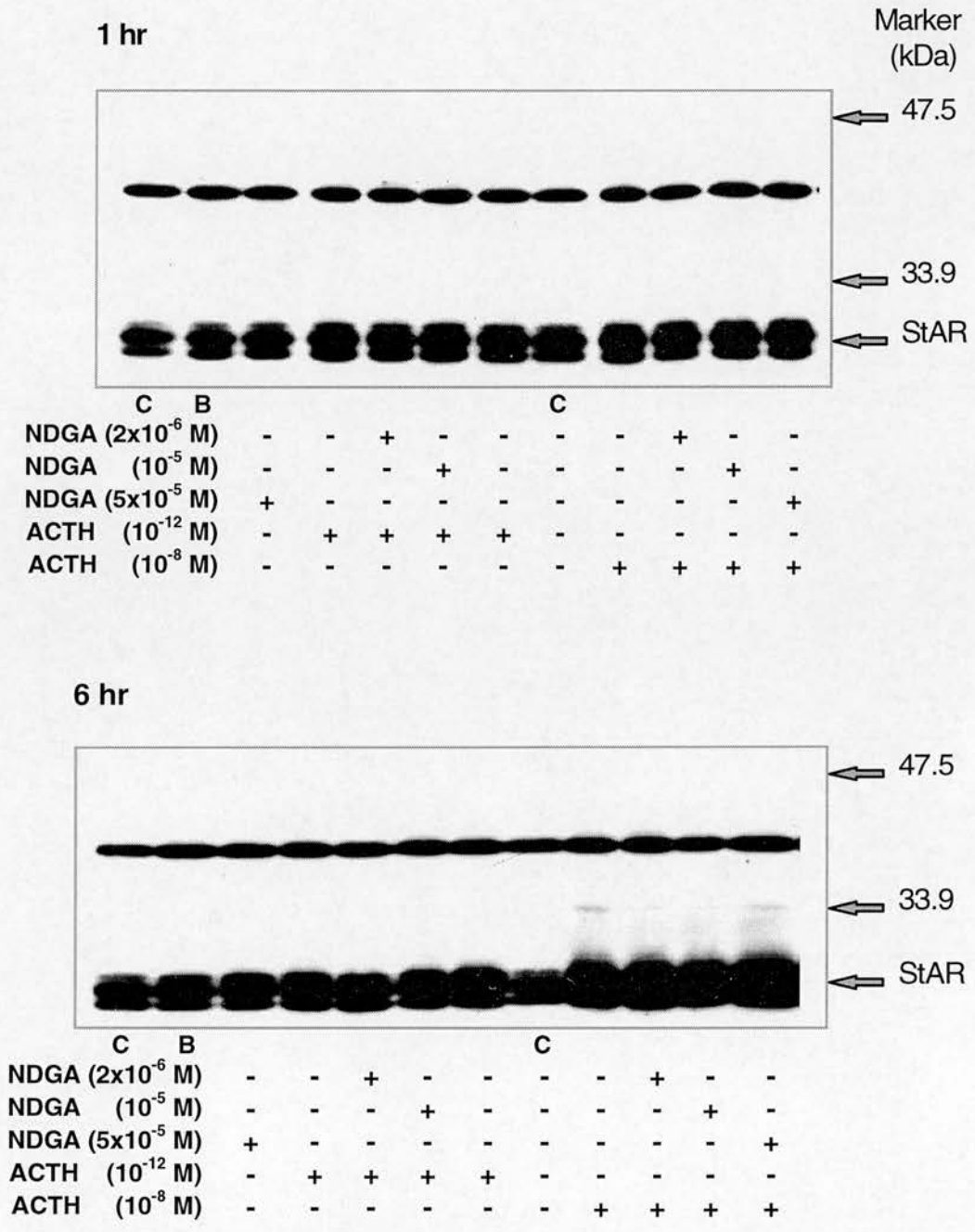

Fig. 5-3-2a Western immunoblots of StAR protein in response to various concentrations of NDGA at 1 hr (upper panel) and at 6 hr (lower panel). C: Zero time point untreated cells. B: Basal (untreated cells at 1 or 6 hr). Samples (25 µg protein) were resolved on 12.5% large SDS-PAGE gels and blotted on to PVDF membranes. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1: 25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

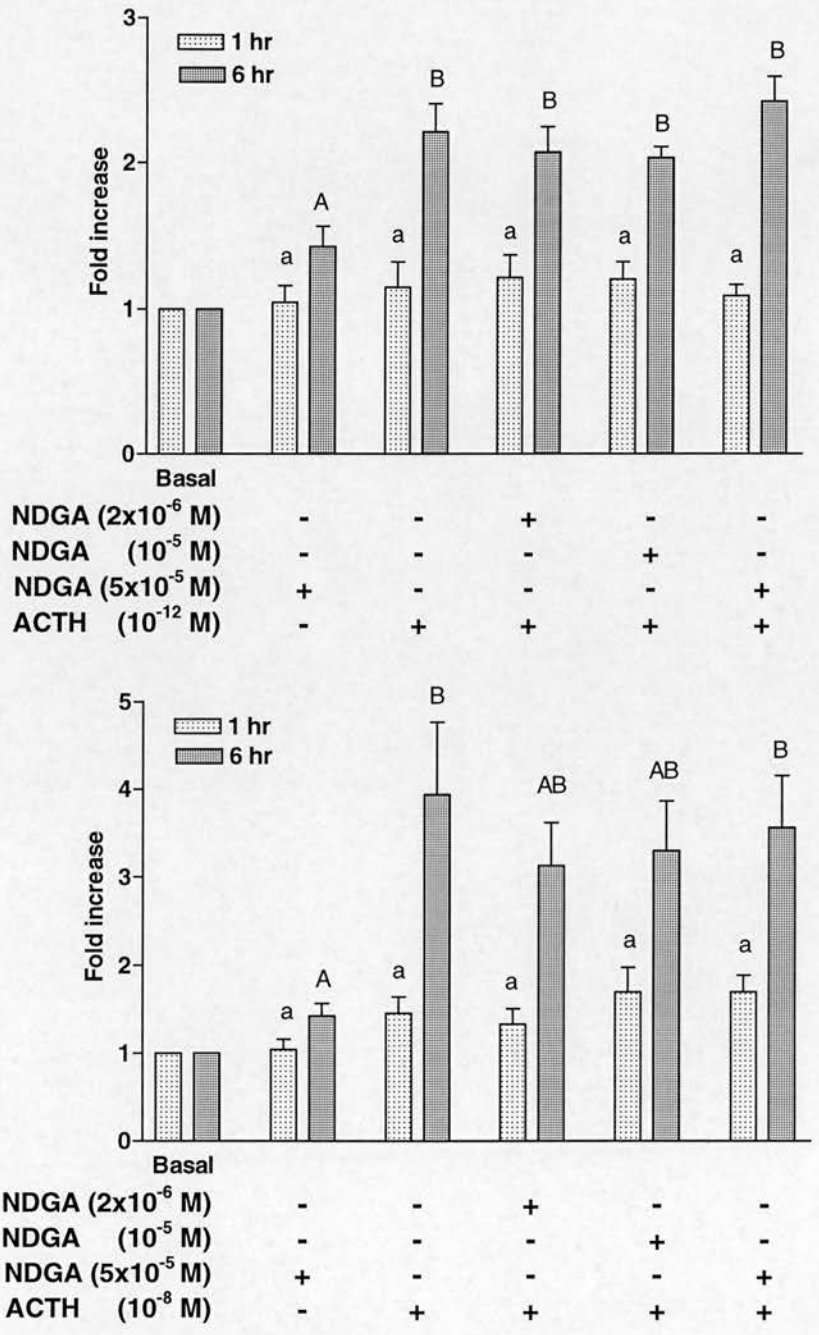

Fig. 5-3-2b Effect of NDGA on StAR protein induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments. Cells were pretreated with various concentrations of NDGA for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

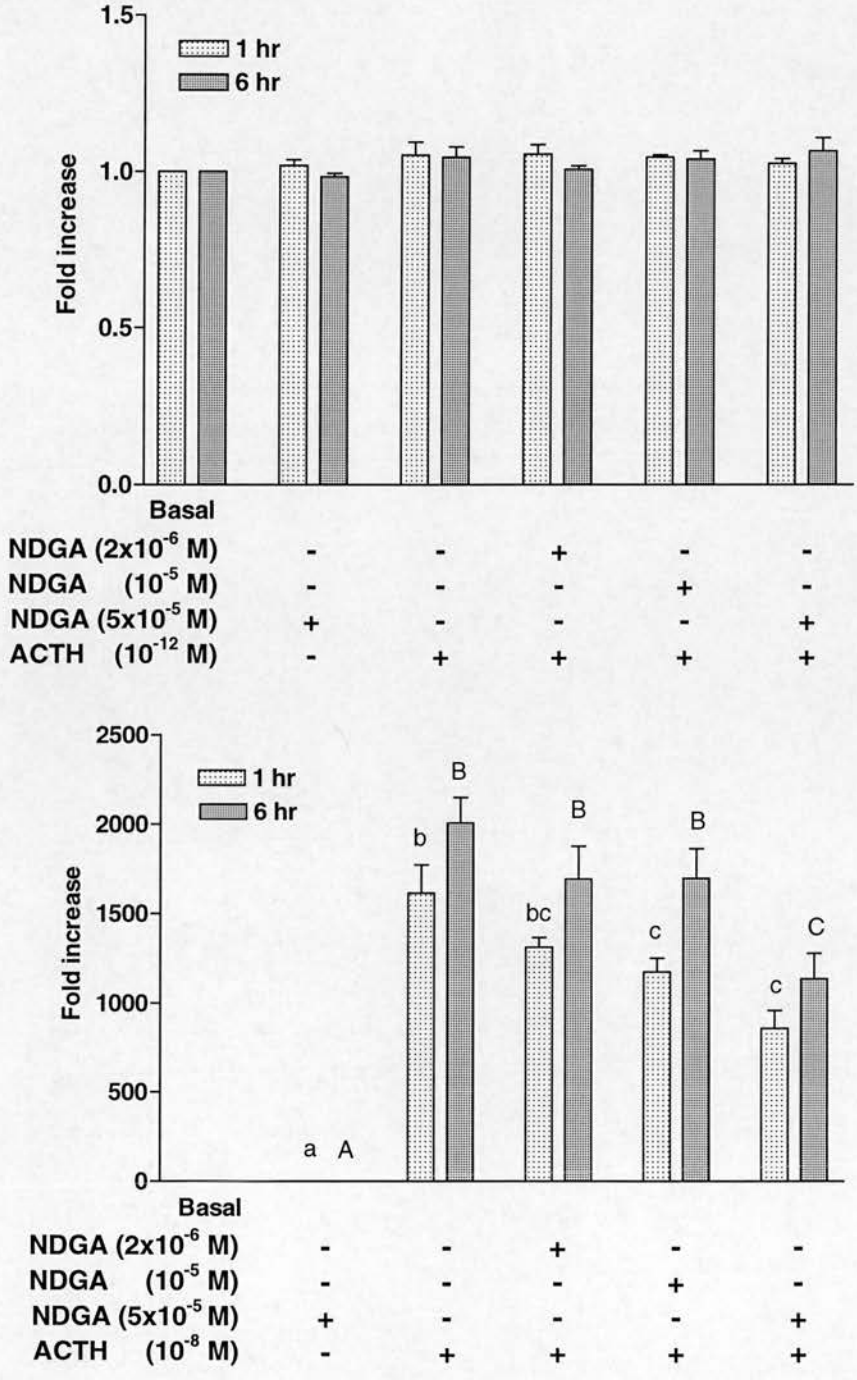

Fig. 5-3-3 Effect of NDGA on cAMP levels induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments. Cells were pretreated with various concentrations of NDGA for 15 min prior to the addition of ACTH (10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

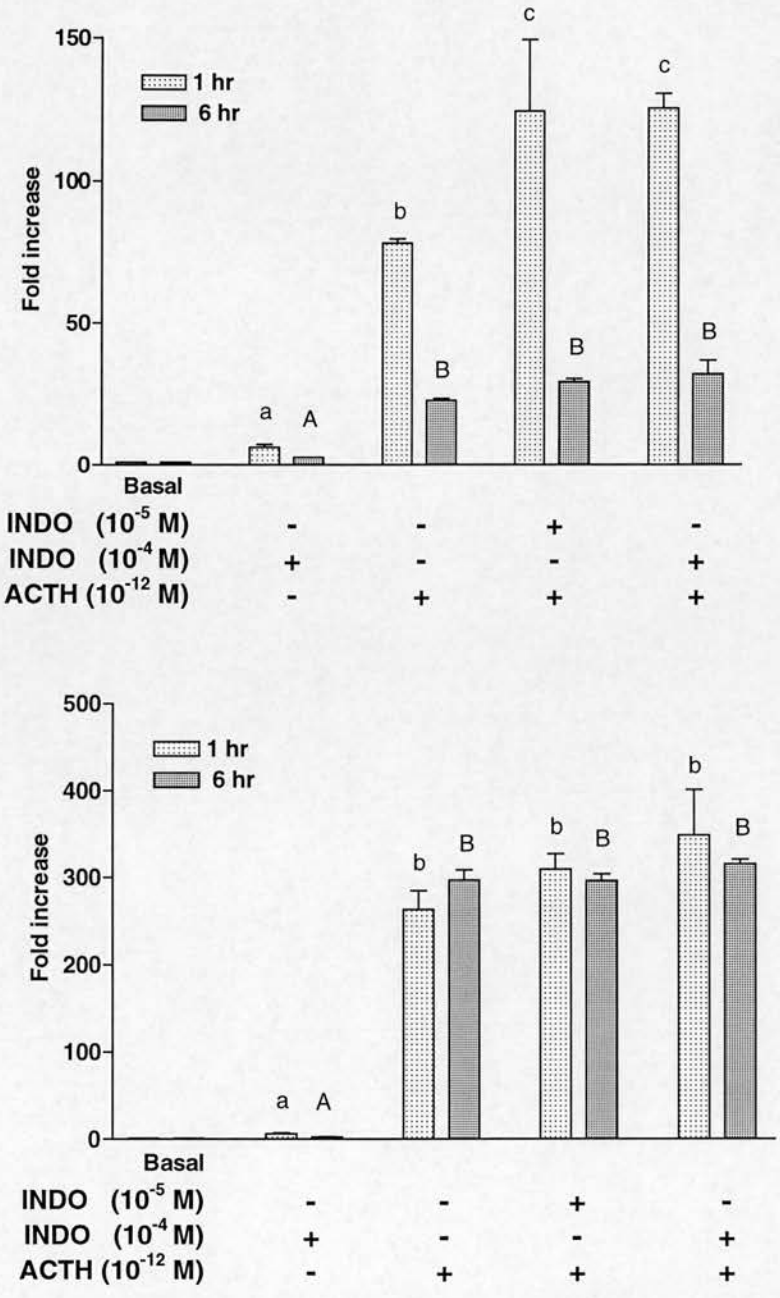

Fig. 5-4-1A Effect of INDO on cortisol levels induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments. Cells were pretreated with different concentrations of INDO for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

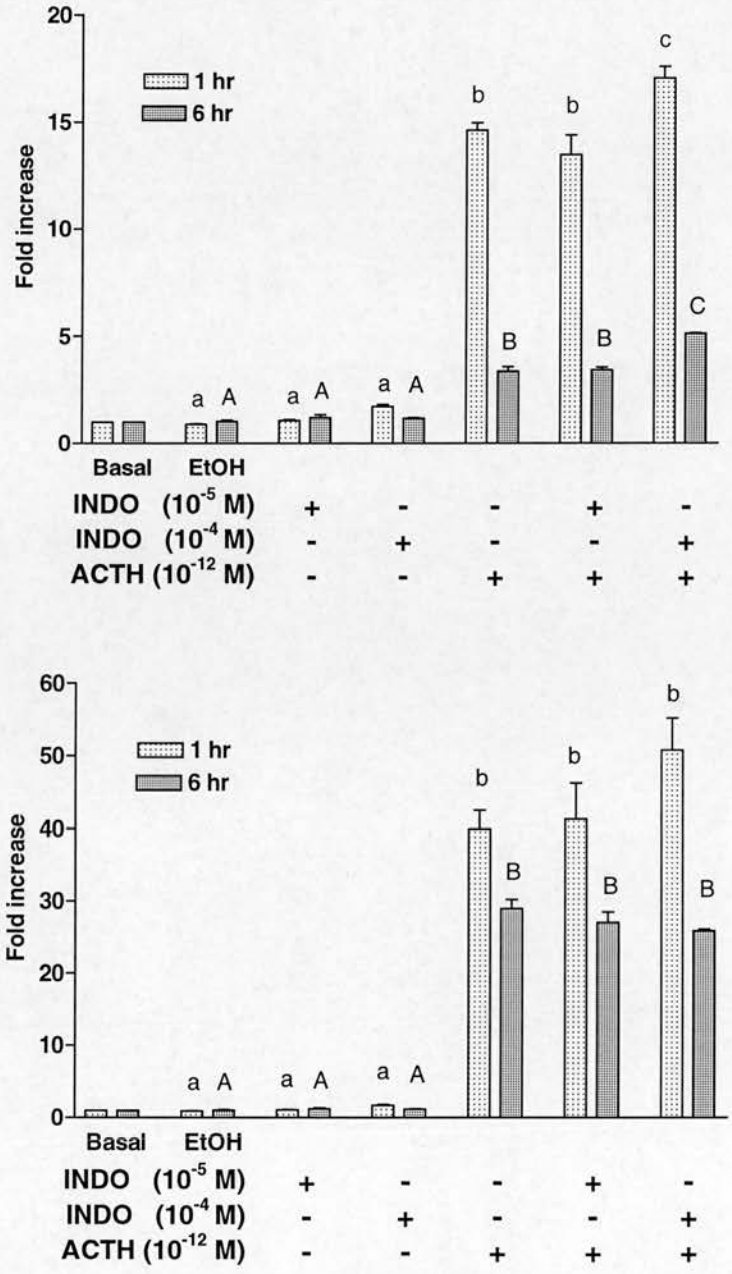

Fig. 5-4-1B Effect of INDO on cortisol levels induced by low-concentration (upper panel) and high-concentration ACTH (lower panel) treatments in FAF BSA medium. Cells were pretreated with INDO for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

In the medium containing 0.2% FAF BSA, a high concentration of INDO (10^{-4} M) enhanced ACTH (10^{-12} M)-induced increases in cortisol levels at both 1 and 6 hr ($P<0.05$ and $P<0.01$ respectively; Fig. 5-4-1B-upper panel). INDO at 10^{-4} M slightly promoted ACTH (10^{-8} M)-stimulated cortisol increase at 1 hr, but did not affect the elevated cortisol level at 6 hr (Fig. 5-4-1B-lower panel).

5.4.2 Effect of INDO on StAR protein level

INDO did not alter 10^{-12} nor 10^{-8} M ACTH-induced changes of StAR protein levels at either 1 hr or 6 hr (Fig. 5-4-2a-upper and lower panels; Fig. 5-4-2b-upper and lower panels).

5.4.3 Effect of INDO on cAMP formation

There was no effect of INDO alone or INDO in the presence of 10^{-12} M ACTH on cAMP production at both 1 and 6 hr treatment periods (Fig. 5-4-3-upper panel). Fig. 5-4-3 (lower panel) showed that INDO at 10^{-5} and 10^{-4} M markedly inhibited ACTH (10^{-8} M) induced-cAMP production ($P<0.05$ and $P<0.01$ respectively) at I hr. Similarly, INDO at 10^{-5} and 10^{-4} M resulted in a decrease in the cAMP levels at 6 hr ($P<0.001$).

5.5 ROLE OF ARACHIDONIC ACID

5.5.1 Effects of arachidonic acid on cortisol output

Exogenous arachidonic acid (10^{-4} M) did not significantly stimulate cortisol secretion but enhanced ACTH (10^{-12} M)-induced cortisol increase at both 1 and 6 hr ($P<0.01$; Fig. 5-5-1-upper panel) and ACTH (10^{-8} M)-induced cortisol levels at 1 hr ($P<0.05$; Fig. 5-5-1-lower panel).

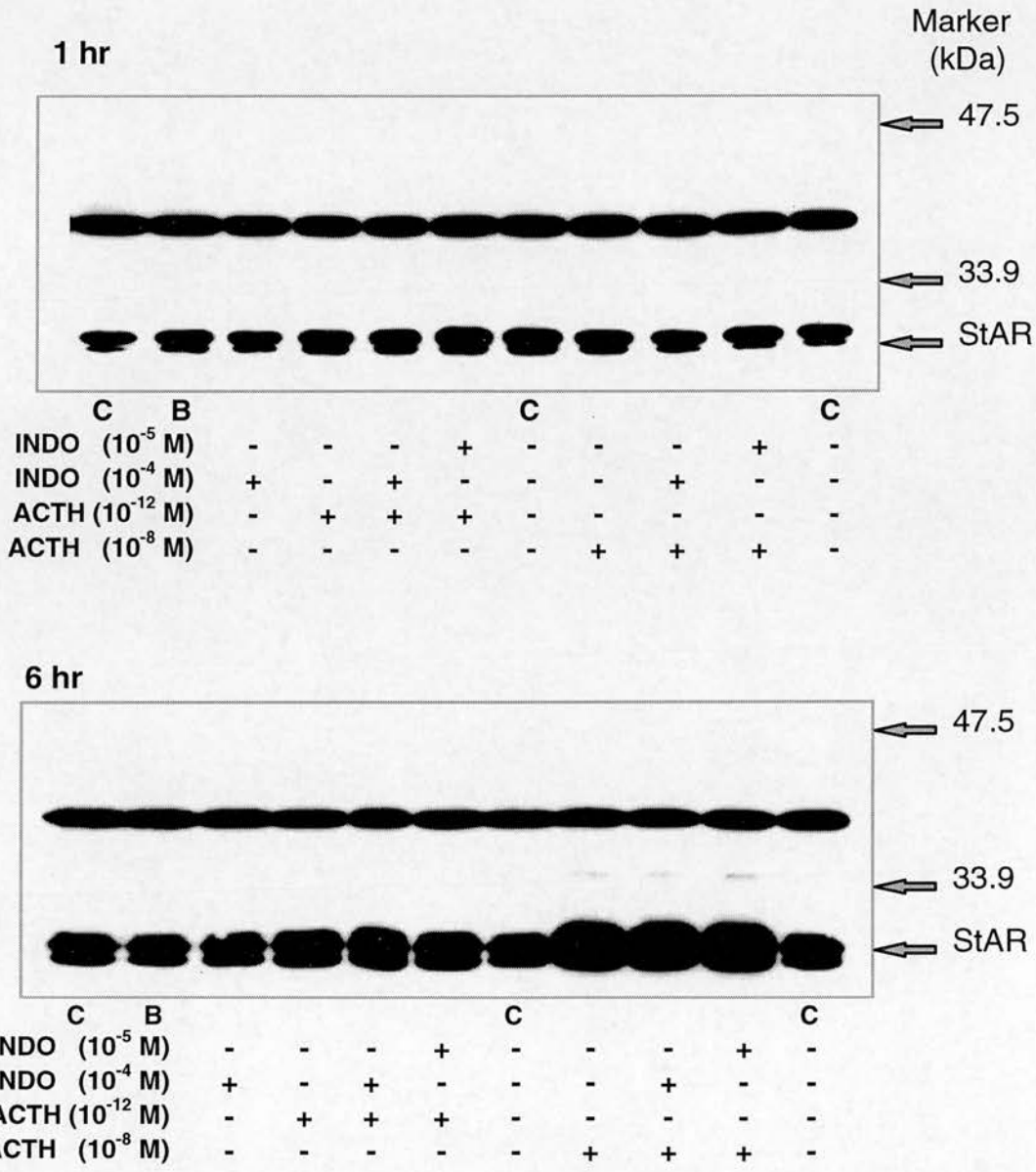

Fig. 5-4-2a Western immunoblots of StAR protein in response to various concentrations of INDO at 1 hr (upper panel) and at 6 hr (lower panel). C: Zero time point untreated cells. B: Basal (untreated cells at 1 or 6 hr). Samples (25 µg protein) were resolved on 12.5% large SDS-PAGE gels. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1: 25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

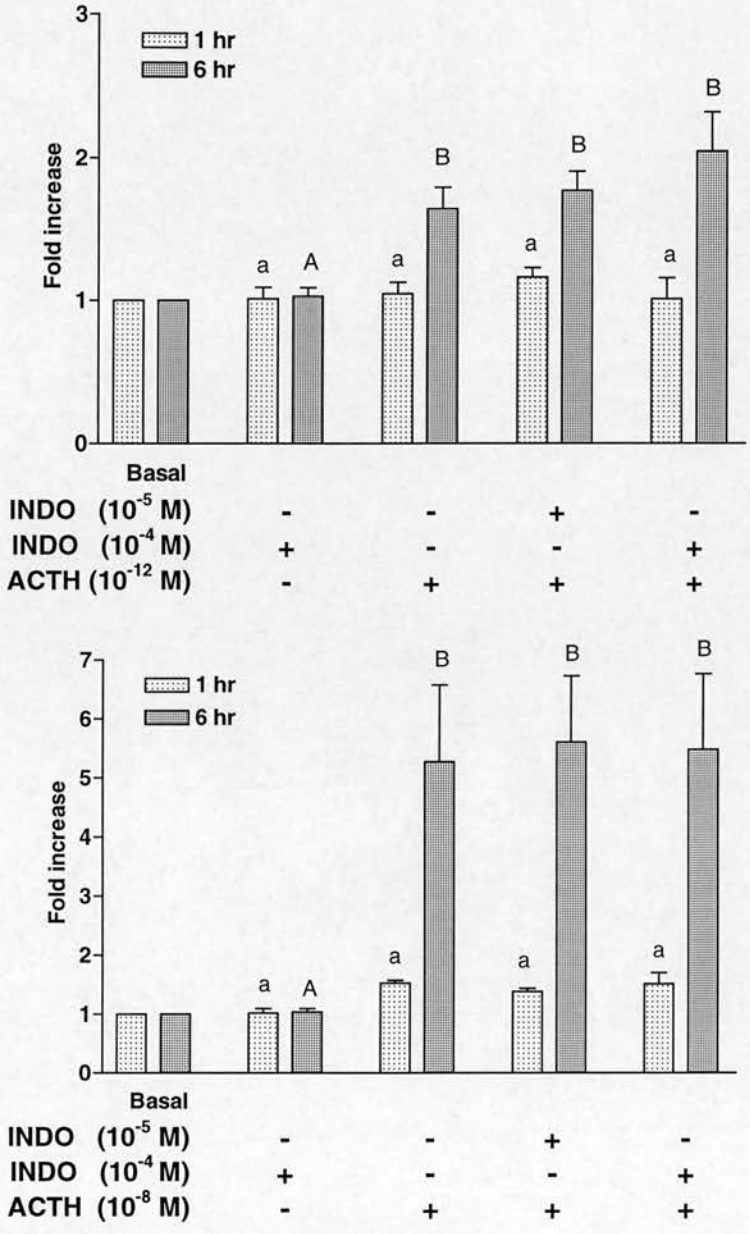

Fig. 5-4-2b Effect of INDO on StAR protein induced by low-concentration (upper panel) and high-concentration ACTH (lower panel) treatments. Cells were pretreated with different concentrations of INDO for 15 min prior to the addition of ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels in a representative experiment performed in triplicate (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

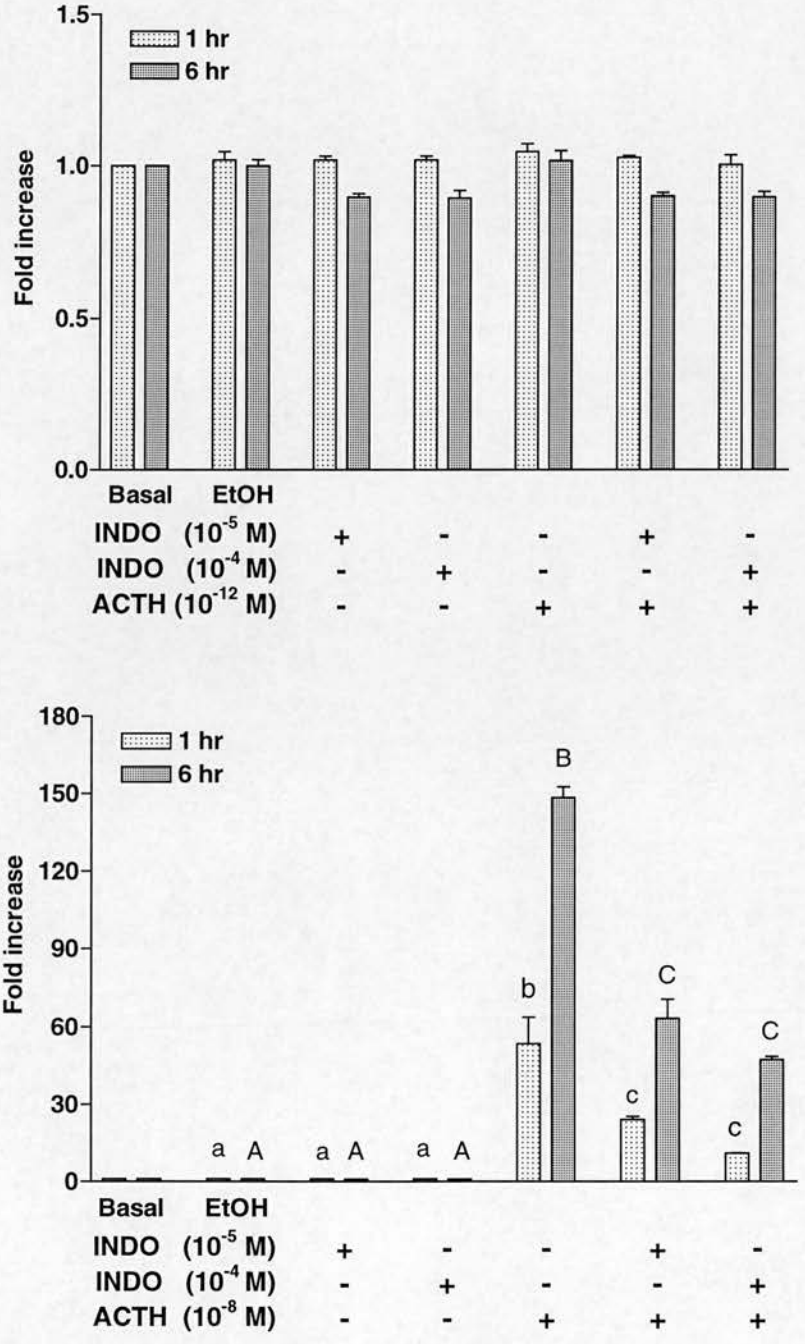

Fig. 5-4-3 Effect of INDO on cAMP levels induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments in FAF BSA medium. Cells were pretreated with INDO for 15 min prior to the addition of ACTH (10^{-12} or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

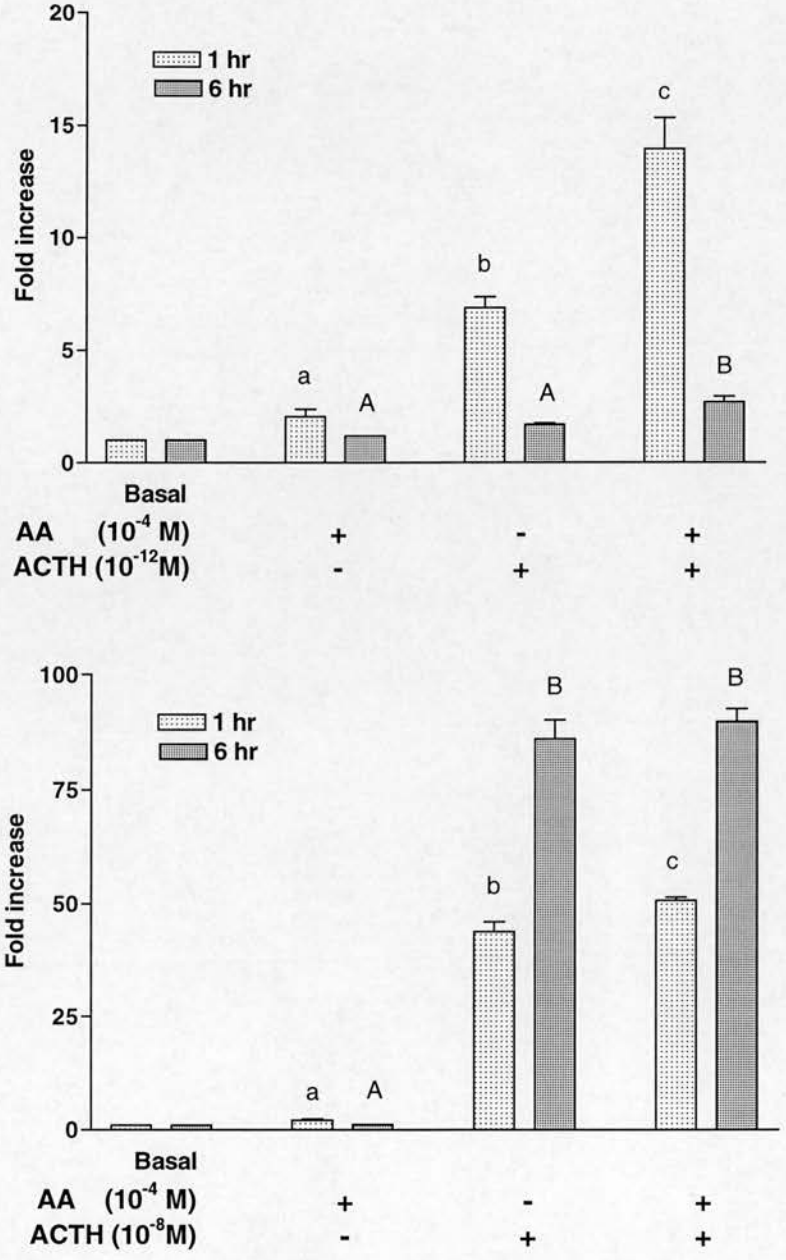

Fig. 5-5-1 Effect of arachidonic acid (AA) on cortisol secretion induced by low-concentration ACTH (upper panel) and high-concentration ACTH (lower panel) treatments in FAF BSA medium. Cells were treated with AA, ACTH and combination of AA and ACTH (10^{-12} M or 10^{-8} M). Values are expressed as fold increase over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time points and letters in upper case indicate 6 hr time points.

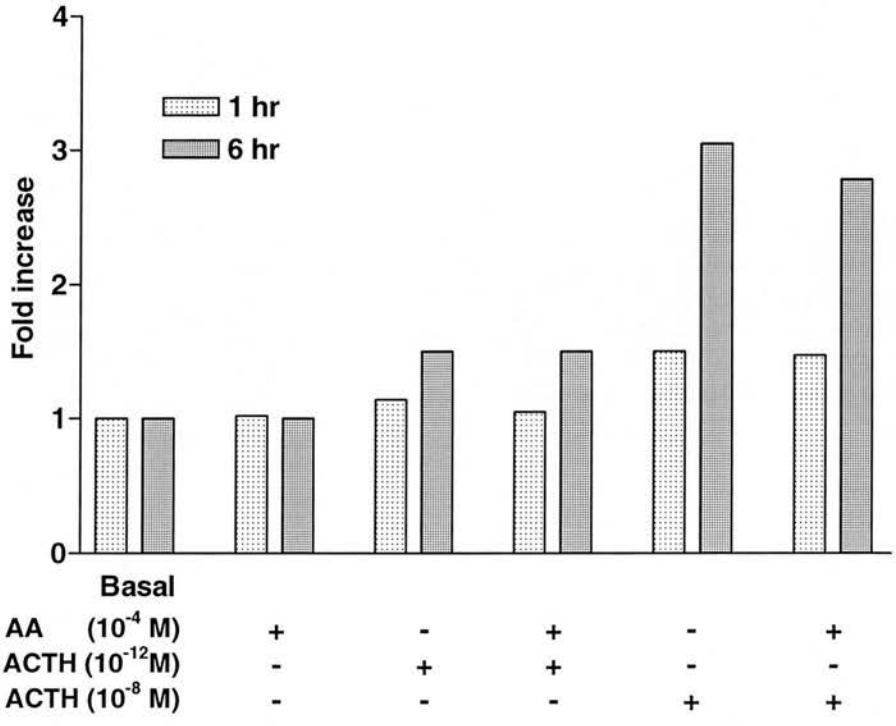

Fig. 5-5-2 Effect of arachidonic acid (AA) on StAR protein induced by low- and high-concentration ACTH treatment in FAF BSA medium. Cells were treated with AA, ACTH (10^{-12} M or 10^{-8} M) and combined AA and ACTH. Values are expressed as fold increase over basal levels in a signal Western immunoblot.

5.5.2 Effect of arachidonic acid on StAR protein

Fig. 5-5-2 showed that AA had no notable effects on the basal and ACTH (10^{-12} and 10^{-8} M)-induced StAR protein levels.

5.6 EXAMINATION OF THE SPECIFICITY OF INHIBITORS

27-Nor-5-cholesten-3β-ol-25-one (25-KETO), a steroidogenic substrate which readily traverses the mitochondrial membrane due to its hydrophilic nature, was used to test the specificity of the compounds described above.

Comparison between 25-KETO alone and 25-KETO in the presence of the compounds showed that 4-BPB (10^{-5} M) and INDO (10^{-5} M) did not change the cortisol levels in response to 25-KETO at both 1 and 6 hr (Fig. 5-6-1). On the other hand, NDGA at $3x10^{-5}$ and $5x10^{-5}$ M significantly reduced the cortisol levels in response to 25-KETO ($P<0.001$ for both tests) at 1 hr and 6 hr ($P<0.05$ and $P<0.01$ respectively; Fig. 5-6-2).

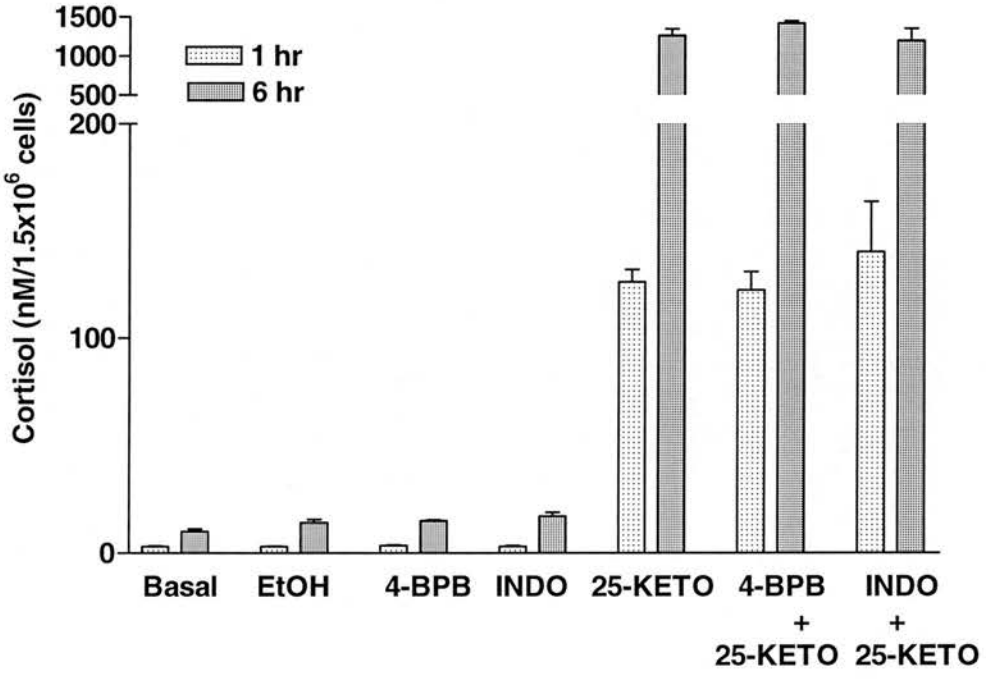

Fig. 5-6-1 Effects of 4-BPB and INDO on cortisol output in response to 27-nor-5-cholesten-3β-ol-25-one (25-KETO) in FAF BSA medium. Cells were pretreated with 4-BPB (10^{-5} M) or INDO (10^{-5} M) for 15 min prior to the addition of 25-KETO ($2x10^{-5}$ M). The media were collected for cortisol assay after 1 hr and 6 hr treatments. Values are expressed as mean ± SEM, n=3. Data (25-KETO vs. 25-KETO plus 4-BPB and 25-KETO vs. 25-KETO plus INDO respectively) were examined by two-tail t test ($P>0.05$ for both tests).

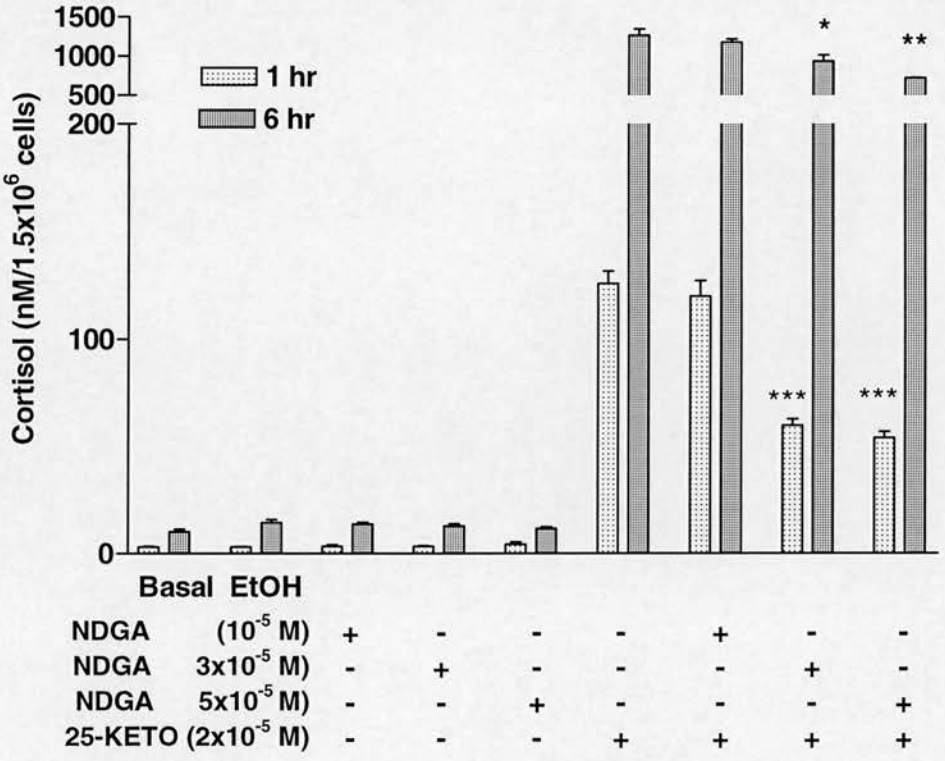

Fig. 5-6-2 Effects of NDGA on cortisol secretion in response to 27-Nor-5-cholesten-3β-ol-25-one (25-KETO) in FAF BSA medium. Cells were pretreated with various concentrations of NDGA for 15 min prior to the addition of 25-KETO ($2x10^{-5}$ M). The media were collected for cortisol assay after 1 hr and 6 hr incubation. Values are expressed as mean ± SEM, n=3. Data (25-KETO vs. 25-KETO plus NDGA) were examined by two-tail t test (*P<0.05, **P<0.01 and ***P<0.001).

5.7 DISCUSSION

The results presented here again clearly demonstrated that 10^{-12} M ACTH induced significant changes in cortisol levels, which was more apparent acutely (1 hr) than that at a relatively more chronic phase (6 hr). However, no notable change in cAMP production by 10^{-12} M ACTH was observed after either 1 or 6 hr ACTH treatment, implying that ACTH-stimulated steroidogenesis may be mediated by other second messenger(s). Previous work has also shown the dissociation between steroidogenesis and cAMP formation. ACTH stimulates steroidogenesis effectively at concentrations much lower than those which produce measurable increases in cAMP output (Beall and Sayers, 1972; Kojima et al., 1985; Perchellet and Sharma, 1979).

The elevation of cortisol induced by low (10^{-12} M) and high (10^{-8} M) concentrations of ACTH was reduced by 4-BPB treatment at 1 hr but not 6 hr, suggesting that PLA_2 may participate in the AA release and the action of PLA_2 may be transient. On the other hand, the expression of StAR protein induced by both 10^{-12} M and 10^{-8} M ACTH was not altered by 4-BPB, suggesting the role of PLA_2 may be independent of newly synthesized StAR protein.

Although the liberation of AA may result principally from the action of PLA_2, other routes such as phospholipase C-AA cannot be excluded (Naor, 1991). Moreover, cholesterol esters may be also a source for AA because the adrenal gland contains cholesterol esters packaged in lipid droplets.

The studies on the downstream AA cascade revealed a complex effect on steroidogenesis in bovine adrenal ZF cells. NDGA severely impaired ACTH-induced cortisol secretion, providing a evidence that LOX pathway of the AA cascade may act as a major second messenger. This agreed with reports by other groups. AA can be transformed into two monohydroxy derivatives: 5- and 15-HETE via 5- and 15-LOX respectively (Omura et al., 1990) and the comparison of the effects of exogenous lipoxygenase metabolites (5-HPETE, 12-HPETE and 15-HPETE) on pregnenolone production showed that 15-HPETE was more significant in enhancing

steroidogenic activity in bovine adrenal ZF cells (Yamazaki et al., 2000), implying that 15-LOX may be a principal form of LOX for AA metabolism.

AA-861, a LOX inhibitor, suppressed the stimulatory effects of ACTH and NPS-ACTH on steroidogenesis without affecting StAR protein contents and the administration of 15-HPETE enhanced the steroidogenic activity, but did not promote the expression of StAR protein. This is suggestive that the action of lipoxygenase-catalyzed products may be via a StAR-independent mechanism in bovine adrenocortical cells (Yamazaki et al., 1996; 1998; 2000). In contrast to these studies, other evidence showed that the AA cascade was correlated with both steroid synthesis and the levels of StAR protein. The effects of AA on LH- and Bt_2cAMP-stimulated progesterone synthesis were regulated by StAR protein; NDGA (70 μM) inhibited the expression of StAR protein and progesterone output in MA-10 Leydig tumor cells (Wang et al., 1999; 2000). These observations are suggestive that the mechanism by which LH-induced progesterone levels in MA-10 Leydig tumor cells might differ from that of ACTH-stimulated cortisol secretion in primary bovine adrenal ZF cells.

The results from this work showed that NDGA did not change the levels of StAR protein in the presence of 10^{-12} M ACTH, whereas NDGA appeared slightly to alter ACTH (10^{-8} M)-induced increases in StAR protein. As the quantitative estimations were ambiguous, more work is required to evaluate the role of StAR protein in this regard.

In some cases, INDO had modest stimulatory effects on 10^{-12} M ACTH-induced cortisol increases, especially in the medium containing the regular BSA. Possible mechanisms by which INDO potentiated ACTH-stimulated steroidogenesis are:

i) Because COX-catalyzed AA conversion is blocked by INDO, more AA (substrate) is redirected to LOX-catalyzed metabolism that may be a major signalling pathway. Equally, AA may be also diverted to the third pathway of the downstream AA cascade and metabolized by cytochrome P450-dependent monooxygenase (MOX) (Nishimura et al., 1989), i.e. INDO may indirectly amplify the effect of MOX on cortisol output.

ii) An increase in cortisol might be caused by abolishing the down-regulation of a signalling pathway. It has been found that PGF_{α} treatment attenuated the levels of StAR mRNA and progesterone in the human corpora lutea (Chung et al., 1998). A speculation is that the negative regulation of PGs on steroidogenesis might be eliminated when the route of PG transformation is blocked.

Yamazaki and co-workers found that INDO (100 μM) enhanced pregnenolone production in bovine adrenal ZFR cells (Yamazaki et al., 1996) and Wang et al showed that INDO (10 μM) promoted Bt_2cAMP-induced progesterone and StAR protein levels in MA-10 Leydig tumor cells (Wang et al., 2000). However, they did not state that whether regular BSA or FAF-BSA was used in these experiments. One should be aware that the regular BSA may lead to an artifact as some unidentified components in this product may mask the effects of inhibitors/compounds on steroidogenesis. This issue is discussed further later.

INDO did not change the levels of StAR protein in bovine adrenal ZF cells. Whereas, the results from MA-10 Leydig tumor cells showed that INDO enhanced the expression of StAR protein. Whether this is due to species specificity or the different mechanism in these two cell types remained to be investigated.

As shown in section 5.4. INDO potentiated or had no effect on ACTH-induced cortisol output, while it potently inhibited cAMP formation under the same conditions. Meanwhile, the examination of inhibitor specificity using 25-KETO demonstrated that INDO did not affect 25-KETO conversion to cortisol, providing evidence that INDO did not cause a nonspecific effect. Therefore, this finding implies that an interaction may exist between AA-COX and cAMP pathways, and also supports the notions on that ACTH-activated cAMP production may exceed the level that is required for steroidogenesis and factor(s) apart from cAMP also serve as second messengers. It has been found that after the cortisol dose-response curve reached a plateau, higher doses of ACTH continued to increase cAMP without further stimulation of steroidogenesis in bovine adrenal ZF cells (Peytremann et al., 1973).

The effects of AA alone on cortisol output and StAR protein were not significant, consistent with the report by Wang et al on MA-10 Leydig tumor cells. The addition of AA did not affect progesterone production nor the expression of StAR protein in MA-10 Leydig tumor cells (Wang et al., 2000). At physiological conditions, free AA levels are very low since the liberated AA is rapidly metabolized to its downstream derivatives (Naor, 1991). Thus, the major role of the AA cascade may be through downstream metabolites rather than AA itself. AA promoted ACTH-induced cortisol increases, especially in the presence of 10^{-12} M ACTH. The mechanism for this stimulatory effect was probably that AA metabolites also mediated steroidogenesis; this may be critical at low concentrations of ACTH.

The effects of ACTH clearly demonstrated that 10^{-8} M ACTH dramatically increased the cAMP levels, leading to marked cortisol output. Meanwhile, the inhibitors of AA cascade also altered 10^{-8} M ACTH-induced cortisol secretion. On the other hand, 10^{-12} M ACTH did not change the cAMP levels and the effects of inhibitors on cortisol levels were more marked compared to those of 10^{-8} M ACTH. In summary, 10^{-8} M ACTH may activate at least two signalling systems, of which cAMP is a predominate pathway; while 10^{-12} M ACTH may activate the PLA_2-AA cascade as a key signaling pathway. Dual signalling systems may co-exist under physiological conditions.

In parallel with a possible connection between COX-AA and cAMP, it appeared a cross-talk between the LOX-AA and the cAMP pathway as NDGA was also able to reduce the cAMP levels. One mechanism of their interaction might be through Ca^{2+}. Ca^{2+} is a potent stimulator of intramitochondrial cholesterol transfer in bovine ZG cells (Cherradi et al., 1996) and it can be enhanced by AA metabolites (Volpi et al., 1980). The action of $cPLA_2$ (a major isoform of PLA_2) in adrenal ZF cells is Ca^{2+}-dependent (Lin et al, 1992). Other potential links may also play roles. For instance, prostacyclin, a product of COX action, elevates cAMP levels in platelets (Gorman et al., 1977). In kidney the AA metabolites by MOX were stimulated by interventions that increased cAMP (Schwartzman et al., 1985).

Moreover, lipomodulin (also known as annexin I), a 40 kDa regulatory protein may also be involved in the AA cascade. Non-phosphorylated lipomodulin is a inhibitor of

PLA_2. After phosphorylation by cAMP-dependent protein kinase lipomodulin loses its inhibitory property, and this feature may regulate the release of AA. Satoh and co-workers has proposed that lipomodulin might act as a mediator between cAMP-PKA and PLA_2 (Satoh et al., 1992).

The effect of the PLA_2 inhibitor, 4-BPB on cortisol levels in medium containing 0.2% FAF BSA was similar to those observed using 0.2% regular BSA, indicating that regular BSA may not markedly affect the upstream AA cascade. Because the effects of NDGA and INDO in FAF-BSA medium appeared somehow different from those in regular BSA, this was suggestive that products associated with regular BSA may complicate the downstream metabolism in the AA cascade. The regular BSA may well contain fatty acids such as oleic acid, linoleic acid and AA which contribute to steroidogenesis by becoming internalized in the cells.

The IC_{50} of NDGA for 15-LOX is $3x10^{-5}$ M (Salari et al., 1984), while the effect of NDGA on the response to 25-KETO showed that $3x10^{-5}$ and $5x10^{-5}$ M NDGA reduced 25-KETO-stimulated cortisol increases. This inhibitory effect suggests that NDGA may also impair the activities of other enzymes that may participate in steroidogenesis. For example, NDGA is also an inhibitor of MOX (Capdevila et al., 1988). The results presented by Nishimura et al showed that epoxyeicosatrienoic acids (EETs), the products of MOX stimulated cortisol production in bovine adrenal ZF cells which may have capacity to metabolize AA via the MOX pathway (Nishimura et al., 1989). In rat ZG cells when the MOX-AA pathway was blocked, AA was converted to COX-AA and LOX-AA products (i.e. PGs and HETEs) respectively (Campbell et al., 1991). AA was rapidly converted to PGs/TXs by COX or LTs by LOX and could also be transformed to epoxyeicosatrienoic acid by the epoxygenase-cytochrome P450 system (Smith, 1989).

AA-861 is a highly selective and potent inhibitor for 5-LOX (Ashida et al., 1983) which is a major form of LOX in the rat adrenal ZF (Hirai et al, 1985), but in the bovine adrenal ZF cell 15-LOX is the principal LOX (Yamazaki et al., 1996). As the current inhibitors for 15-LOX lack sufficient specificity, the role of 15-LOX in bovine ZF cells can only be evaluated further when a potent specific inhibitor is available.

Collectively, 10^{-8} M ACTH may trigger both cAMP and AA cascade signalling systems, whereas, 10^{-12} M ACTH activates AA cascades as major mediators for steroidogenesis. ACTH-induced steroidogenesis may be up-regulated by LOX-AA pathway and also through MOX-AA, while it appears to be down-regulated or not affected by the COX-AA pathway. Furthermore, cAMP and the AA cascade might interact via other signalling molecules. The synthesis of StAR protein was regulated by the cAMP pathway, but appeared not to be by AA cascades in bovine adrenal ZF cells.

CHAPTER 6 ROLE OF PROTEIN KINASE C IN MODULATING STEROIDOGENESIS AND StAR PROTEIN

6.1 INTRODUCTION

In response to ACTH binding, second messengers exert their biological effects by activating appropriate protein kinases. Nishikawa and co-workers have demonstrated that 10^{-7} M ACTH can enhance the expression of StAR protein as well as cortisol synthesis via both PKA- and PKC-dependent processes in bovine adrenal ZF cells (Nishikawa et al., 1996). On the other hand, 10^{-12} M ACTH stimulated pregnenolone production without increasing PKA activity (Yamazaki et al., 1996) and the dose-response curve of PKA activity showed that the level of PKA activity was very low at 10^{-12} M ACTH, attaining a maximum at 10^{-9}-10^{-8} M ACTH in bovine adrenal ZF cells (Enyeart and Enyeart, 1998).

The calcium-activated, phospholipid-dependent PKC has been found in bovine adrenal cortex (Vilgrain et al., 1984). The potent phorbol ester, 12-myristate-13-acetate (PMA), also known as 12-*O*-tetrasecanoylphorbol-13-acetate (TPA), is a well characterized positive regulator of PKC (Ashendel, 1985; Castagna et al., 1982). Treatment with PMA and A23187 calcium ionophore promoted steroid hormone synthesis in bovine adrenal ZF cells (Finn et al., 1988; Kenyon et al., 1988). The fatty acids released from phospholipids may participate in the activation of PKC, an integral part of the signal-induced degradation cascade that is initiated by the actions of PLC and PLA_2 (Shinomura et al., 1991). The protein kinase C, isoform α (PKCα) which is present in all cells and tissues so far examined is activated by high concentrations of AA in the presence of Ca^{2+}. AA has been shown to be an activator of PKC in a dose- and calcium-dependent fashion in human neutrophils (McPhail, 1984). Both *in vivo* and *in vitro* studies suggest that AA and DAG act synergistically to activate PKC (Lester et al., 1991) and the biological functions of target proteins can be modulated by PKC in a rapid and reversible manner (Azzi et al., 1992).

The results presented in Chapter 5 have demonstrated that the AA cascade may also mediate steroidogenesis, especially for a physiological concentration of ACTH. Therefore, the aims of this work were:

- To examine whether cortisol output and StAR protein levels are modulated by PMA treatment in the absence and presence of ACTH.
- To evaluate the role of PKC in high- and low-concentration ACTH-stimulated steroidogenesis and its relationship to StAR protein.

6.2 EFFECTS OF PHORBOL ESTER ON CORTISOL SECRETION, StAR PROTEIN AND cAMP FORMATION

6.2.1 Effects of PMA alone and combined PMA with ACTH on cortisol secretion

At 6 hr, PMA (10^{-8}-10^{-6} M) alone had a modest stimulatory effect on cortisol output; the cortisol levels were elevated by 10^{-8} M PMA ($P<0.01$) and 10^{-6} M PMA ($P<0.01$); and peaked at 10^{-7} M PMA ($p<0.001$). At 24 hr, PMA (10^{-8} and 10^{-6} M) appeared to slightly reduce cortisol levels, while 10^{-7} M PMA had no effect (Fig. 6-2-1-upper panel).

PMA (10^{-8}-10^{-6} M) did not alter ACTH (10^{-8}M)-induced cortisol increases after a 6 hr treatment, whereas cortisol output was inhibited in a concentration-dependent manner after prolonged treatment (24 hr, Fig. 6-2-1-lower panel). The detailed comparison is listed in Table 6-2-1.

Table 6-2-1 Multiple comparison tests of the effects of PMA on ACTH (10^{-8} M)-induced cortisol output

Comparisons	P value
ACTH vs. ACTH + PMA (10^{-8} M)	$P<0.001$
ACTH vs. ACTH + PMA (10^{-7} M)	$P<0.001$
ACTH vs. ACTH + PMA (10^{-6} M)	$P<0.001$
ACTH + PMA (10^{-8} M) vs. ACTH + PMA (10^{-7} M)	$P<0.001$
ACTH + PMA (10^{-8} M) vs. ACTH + PMA (10^{-6} M)	$P<0.001$
ACTH + PMA (10^{-7} M) vs. ACTH + PMA (10^{-6} M)	$P<0.05$

6.2.2 Effects of PMA alone and in combination with ACTH on StAR protein levels

The data presented in Fig 6-2-2a&b illustrate that 10^{-8} M and 10^{-7} M PMA slightly enhanced StAR protein levels; 10^{-6} M PMA significantly increased StAR protein

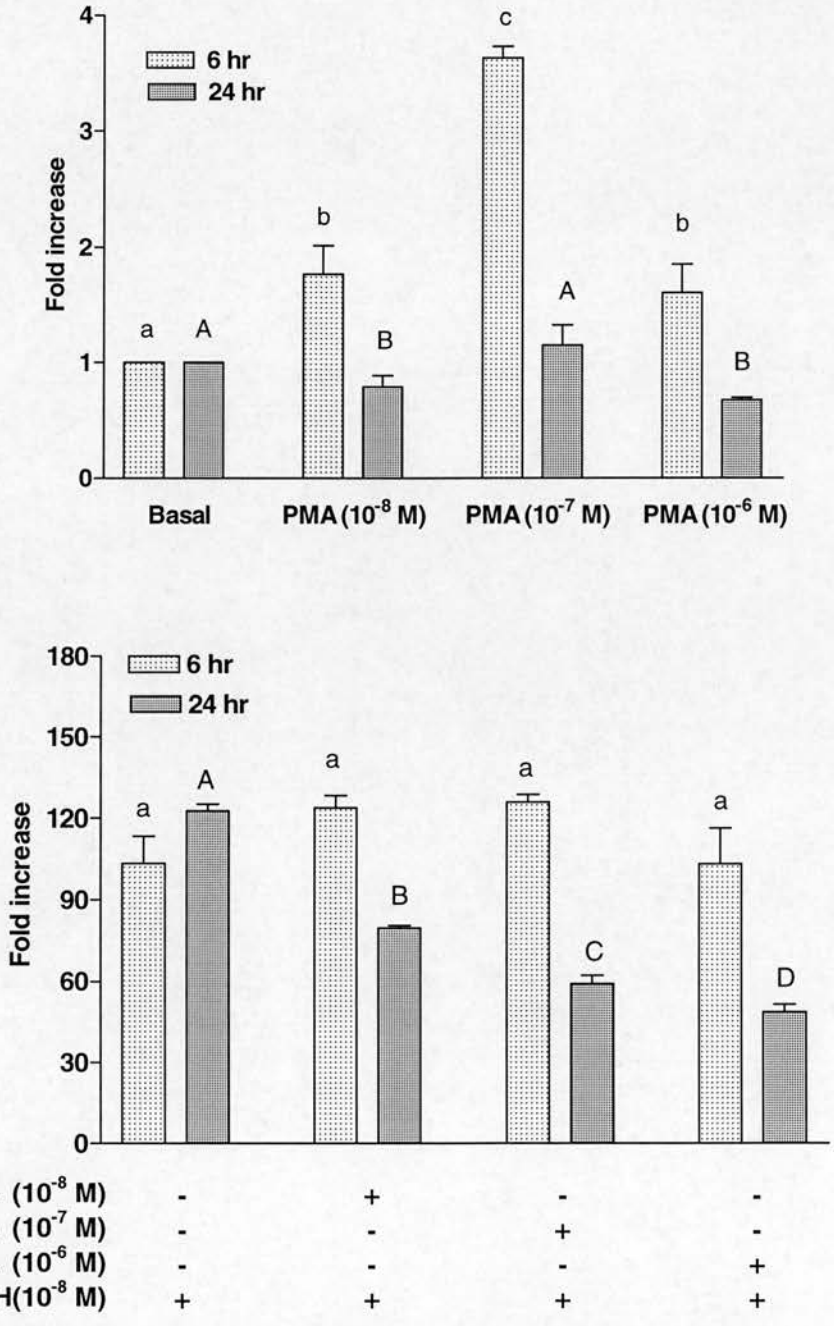

Fig. 6-2-1 Changes of cortisol production in response to PMA (upper panel), ACTH and the combined PMA and ACTH (lower panel) treatments. Cells were treated with PMA (10^{-8}-10^{-6} M), ACTH (10^{-8} M) and combined various concentrations of PMA with ACTH. Values are expressed as fold increases (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate data from 6 hr time points and letters in upper case indicate 24 hr time points.

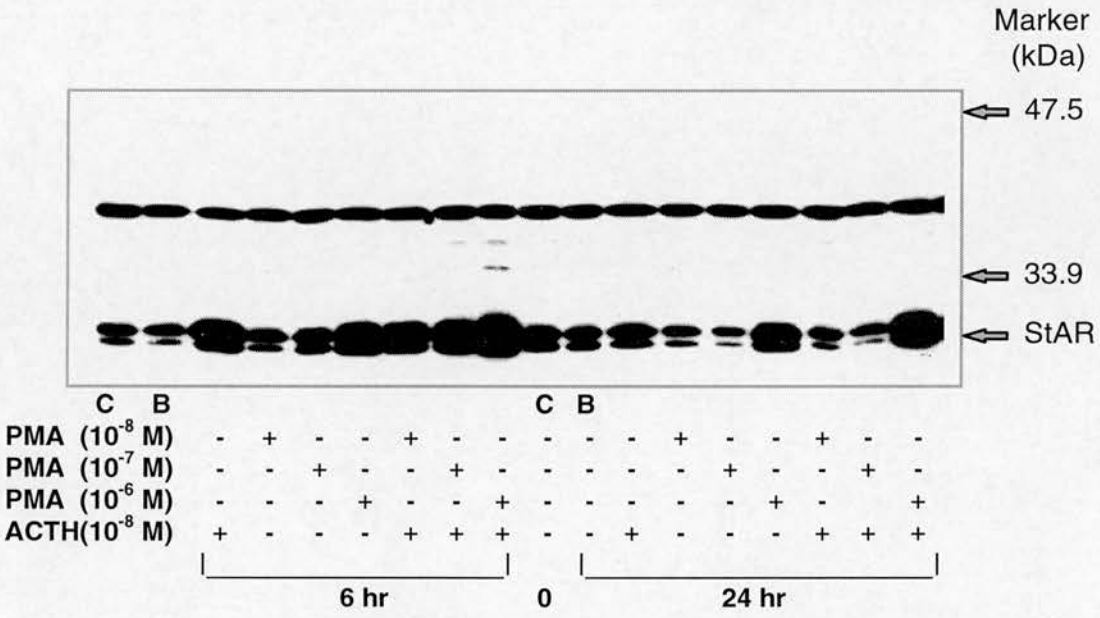

Fig. 6-2-2a Western immunoblot of StAR protein in response to PMA and ACTH. C: Zero time untreated cells; B: Basal (untreated cells at 6 or 24 hr). All samples (25 µg protein) were resolved on a 12.5% large SDS-PAGE gel. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1: 25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

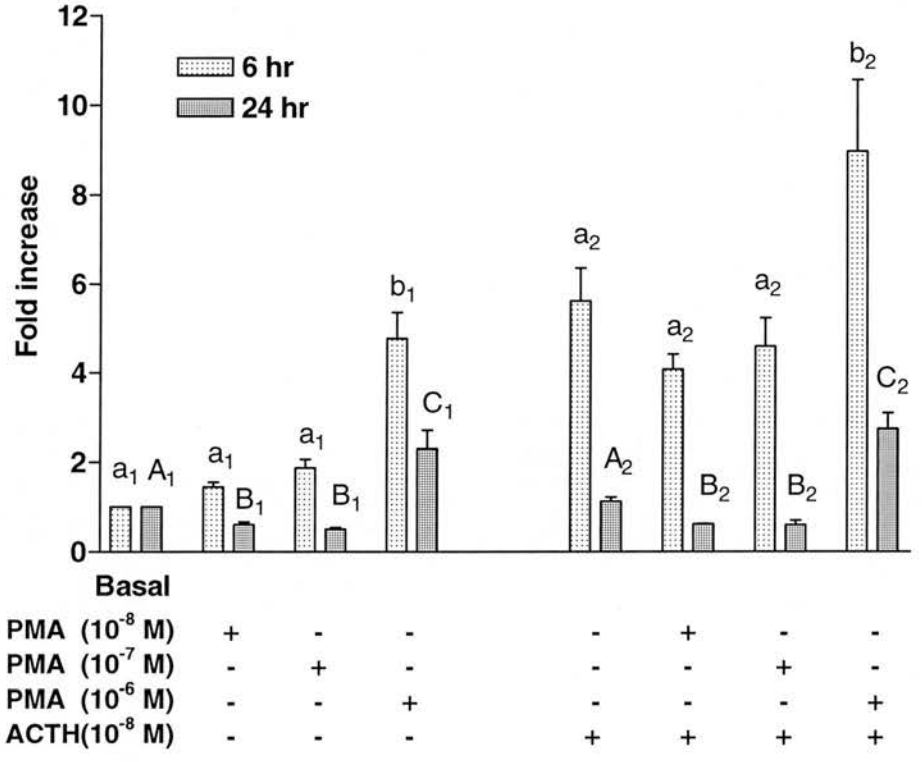

Fig. 6-2-2b The levels of StAR protein in response to PMA, ACTH and the combined PMA and ACTH treatments. Cells were treated with PMA (10^{-8}-10^{-6} M), ACTH (10^{-8} M) and combined ACTH with various concentrations of PMA. Values are expressed as fold increases (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 6 hr time point data and letters in upper case indicate 24 hr time point data. Columns with subscript "1": Multiple comparison between various concentrations of PMA alone and the basal; columns with subscript "2" are multiple comparison between ACTH and ACTH in presence of PMA.

levels ($P<0.05$) and also potentiated ACTH (10^{-8}M)-induced StAR increases ($P<0.01$) after 6 hr treatment. PMA (10^{-8} and 10^{-7} M) appeared to inhibit both the basal and ACTH-promoted StAR protein levels; prolonged 10^{-6} M PMA treatment (24 hr), however, potentiated ACTH-induced StAR levels ($P<0.001$).

The effects of PMA on StAR protein levels were also examined by using 4α-Phorbol-12, 13-didecanoate (4α-PDD), an inactive phorbol ester. As shown in Fig. 6-2-2c&d, 4α-PDD did not affect StAR protein levels either 6 hr or 24 hr treatment periods.

6.2.3 Effects of PMA alone and in combination with ACTH on cAMP accumulation

PMA (10^{-8}-10^{-6} M) alone did not alter cAMP production after both 6 and 24 hr treatment (Fig. 6-2-3-upper panel). PMA at 10^{-8} M significantly potentiated ACTH (10^{-8} M)-induced cAMP increase ($P<0.05$), and also at 10^{-7} and 10^{-6} M ($P<0.001$ for both tests) at 6 hr. Only 10^{-7} PMA significantly enhanced ACTH-induced cAMP level ($P<0.05$) at 24 hr (Fig. 6-2-3-lower panel).

6.3 ROLES OF PROTEIN KINASE C IN MODULATING STEROIDOGENESIS AND StAR PROTEIN

Bisindolymaleimide I (GF109203X), a highly selective, cell-permeant PKC inhibitor was used to evaluate the role of PKC in cortisol output and the level of StAR protein.

The data presented in Fig. 6-3-1a (left panel) showed GF19203X at 10^{-7} and 10^{-6} M had no effect on the basal level of cortisol secretion, while it inhibited cortisol output at 10^{-5} M after 1 hr treatment ($P<0.001$). A similar profile was obtained after 6 hr treatment ($P<0.001$).

Various concentrations of GF109203X (10^{-7} -10^{-5} M) significantly reduced ACTH (10^{-12} M)-induced cortisol increases ($P<0.001$ for all three tests) at 1 hr. GF109203X

(10^{-6} and 10^{-5} M) treatment also inhibited ACTH (10^{-12} M)-elevated cortisol levels ($P<0.05$ and $P<0.001$ respectively) at 6 hr (Fig. 6-3-1a-right panel).

As shown in Fig. 6-3-1b (right panel), GF109203X (10^{-7}-10^{-5} M) attenuated ACTH (10^{-8} M)-stimulated cortisol increases in a concentration-dependent manner after 1 hr treatment, while only 10^{-5} M GF109203X inhibited cortisol output after 6 hr treatment. The multiple comparison is summarized in Table 6-3-1. In addition, GF109203X (10^{-7}-10^{-5} M) appeared not alter the level of StAR protein (Fig. 6-3-2).

Table 6-3-1Multiple comparison tests of the effects of GF109203X on ACTH (10^{-8} M)-induced cortisol output

	1 hr	6 hr
Comparisons	**P value**	**P value**
ACTH vs ACTH + GF109203X (10^{-7} M)	P<0.001	P>0.05
ACTH vs ACTH + GF109203X (10^{-6} M)	P<0.001	P>0.05
ACTH vs ACTH + GF109203X (10^{-5} M)	P<0.001	P<0.01
ACTH + GF109203X (10^{-7} M) vs ACTH + GF109203X (10^{-6} M)	P<0.01	P>0.05
ACTH + GF109203X (10^{-7} M) vs ACTH + GF109203X (10^{-5} M)	P<0.001	P<0.001
ACTH + GF109203X (10^{-6} M) vs ACTH + GF109203X (10^{-5} M)	P<0.001	P<0.01

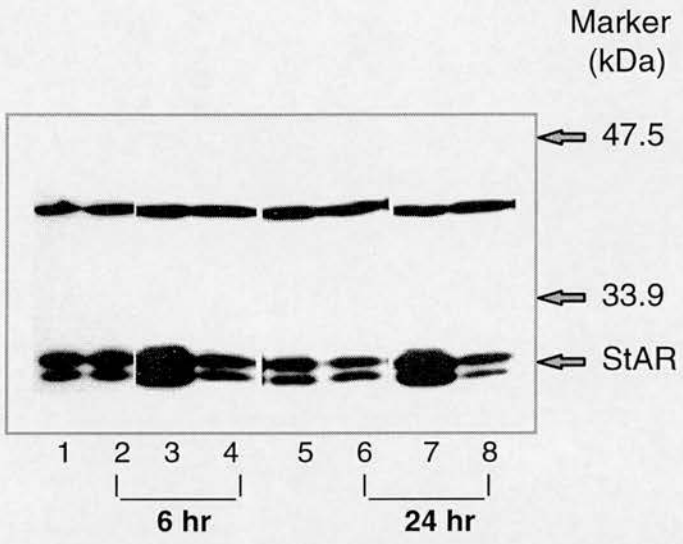

Fig 6-2-2c Western immunoblot of StAR protein in response to PMA and 4α-PDD. Lanes 1 and 5: Zero time untreated cells; lanes 2 and 6: 6 and 24 hr untreated cells respectively; lanes 3 and 7: PMA (10^{-6} M)-treated cells; lanes 4 and 8: 4α-PDD (10^{-6}M)-treated cells. All samples (20 µg protein) were resolved on a 12.5% large SDS-PAGE gel. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

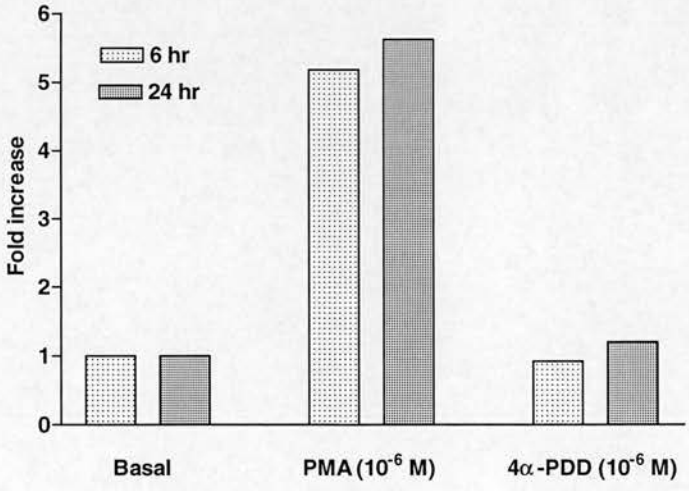

Fig. 6-2-2d Effects of PMA and 4α-PDD on the levels of StAR protein. Cells were treated with PMA (10^{-6} M) and 4α-PDD (10^{-6} M) and harvested for Western immunoblot after 6 and 24 hr treatment. Values from a single experiment are expressed as fold increases.

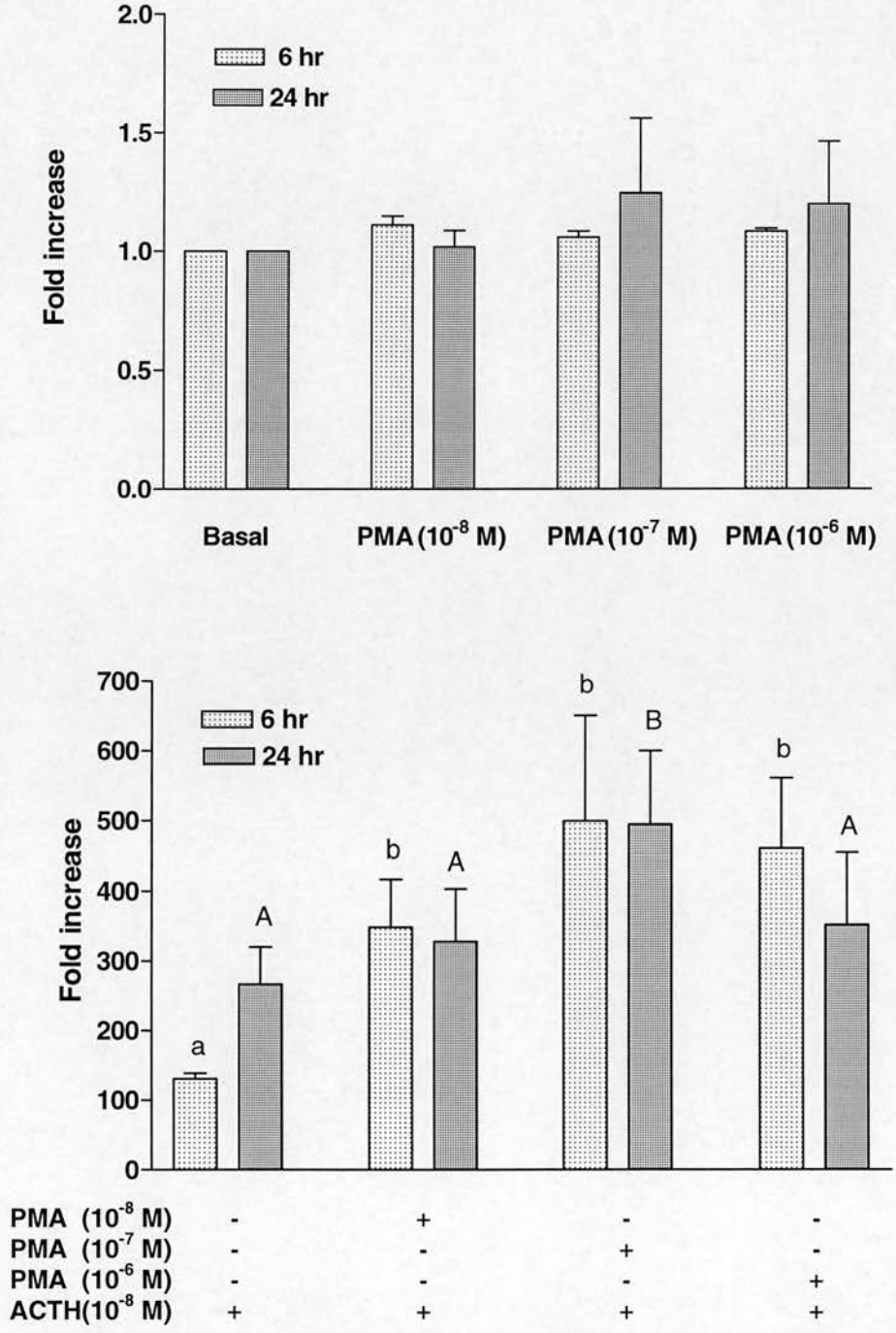

Fig. 6-2-3 Changes of cAMP production in response to PMA (upper panel), ACTH and the combined PMA and ACTH (lower panel) treatment. Cells were treated with PMA (10^{-8}-10^{-6} M), ACTH (10^{-8} M) and combined various concentrations of PMA with ACTH. Values are expressed as fold increases over the basal (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 6 hr time point data and letters in upper case indicate 24 hr time point data.

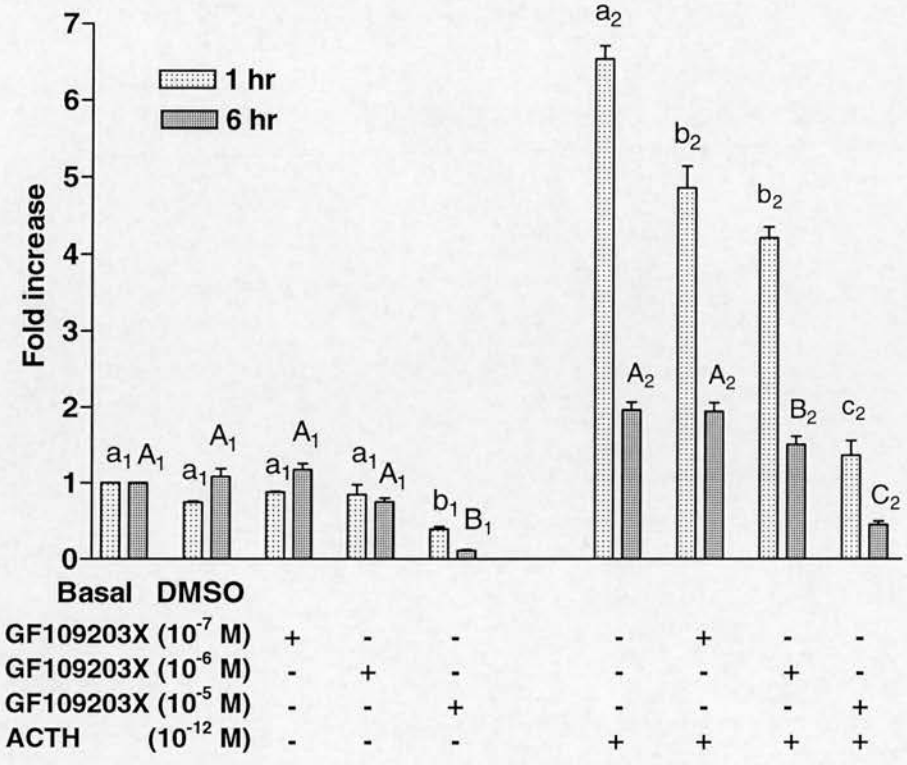

Fig. 6-3-1a Effect of GF109203X on the basal and ACTH (10^{-12} M)-induced cortisol levels. Cells were treated with various concentrations of GF109203X for 60 min prior to the addition of ACTH. Values are expressed as fold increases over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time point data and letters in upper case indicate 6 hr time point data. Columns with subscript "1" are multiple comparison between various concentrations of GF109203X alone and the basal; columns with subscript "2" are multiple comparison between ACTH and ACTH in presence of GF109203X .

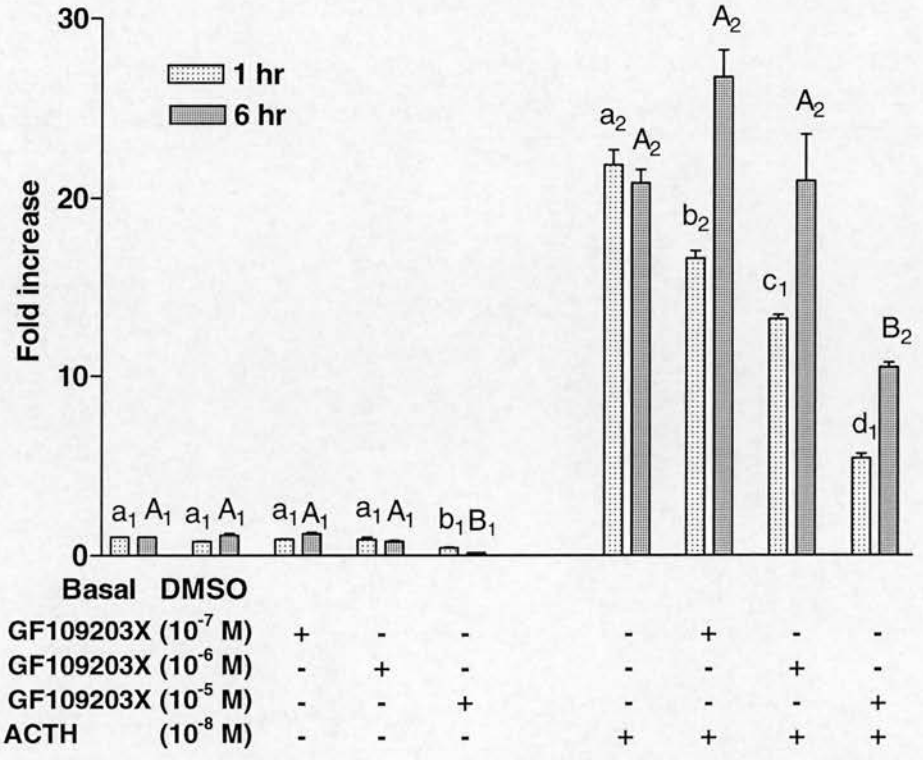

Fig. 6-3-1b Effect of GF109203X on the basal and ACTH (10^{-8} M)-induced cortisol levels. Cells were treated with various concentrations of GF109203X for 60 min prior to the addition of ACTH. Values are expressed as fold increases over basal levels (mean ± SEM), n=3. Columns without the same superscript are significantly different. Letters in lower case indicate 1 hr time point data and letters in upper case indicate 6 hr time point data. Columns with subscript "1" are multiple comparison between various concentrations of GF109203X alone and the basal; columns with subscript "2" are multiple comparison between ACTH and ACTH in presence of GF109203X .

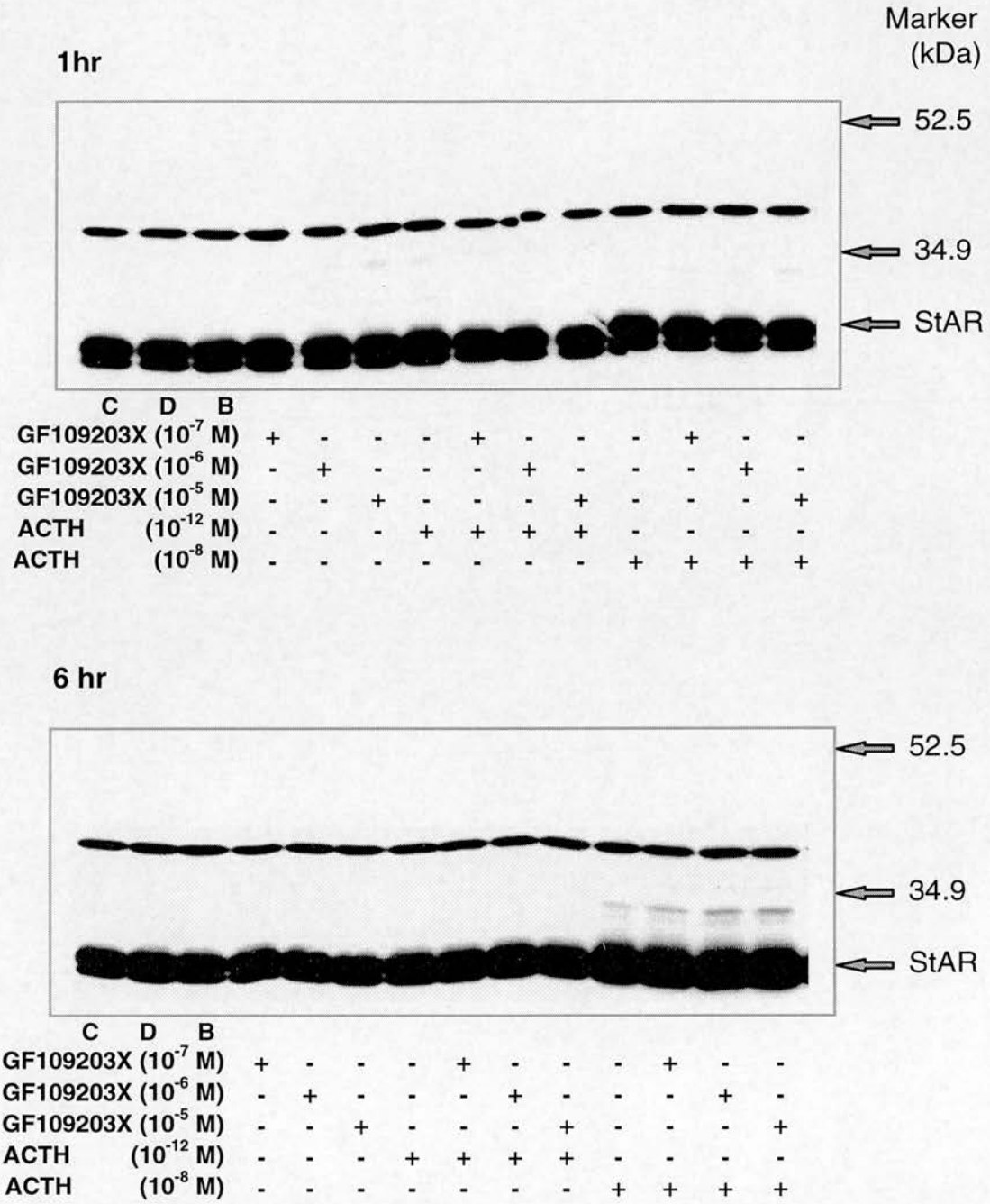

Fig. 6-3-2 Western immunoblots of StAR protein in response to ACTH and various concentrations of GF109203X at 1 hr (upper panel) and at 6 hr (lower panel). C: Zero time point control; D: 0.05% DMSO control; B: 1 hr or 6 hr Untreated cells. Samples (25 µg protein) were resolved on two 12.5% large SDS-PAGE gels. Sheep anti-bovine peptide antibody (1:10,000) and donkey anti-sheep/goat antibody conjugated with HRP (1:25,000) were diluted in PBS/10% Pierce blocking buffer containing 2% milk.

6.4 DISCUSSION

Despite promoting StAR protein synthesis PMA singularly did not markedly increase the cortisol levels. A similar result was obtained by Clark et al who showed that PMA stimulated StAR expression but not steroidogenesis in H295R cells (Clark et al., 1995a). This is possibly due to a lack of Ca^{2+} mobilization to play a synergetic role since PMA requires Ca^{2+} ionophore to open the Ca^{2+} channel for steroidogenesis (Berridge et al, 2000).

PMA (10^{-8}-10^{-6} M) inhibited ACTH-induced cortisol secretion in a concentration-dependent manner after 24 hr treatment. A possible reason for this is that chronic treatment may alter expression of various steroidogenic enzymes, leading to decrease in cortisol production. For example, PMA (10^{-7} M) attenuated the large increase in CYP17 induced by ACTH (10^{-8} M) after prolonged treatment (Bird et al., 1993). This may be a mechanism by which PMA reduced the remarkable increase in ACTH-induced cortisol output although it enhanced StAR protein under the same condition.

PMA, most likely as a PKC activator, also potentiated ACTH-induced cAMP formation, in agreement with other reports. PMA promoted ACTH-stimulated cAMP production on Day 0, 3, and 7 in bovine adrenocortical cells (Begeot et al., 1988) and also in bovine ZG cells (Baukal et al., 1994). The effect of PMA on cAMP levels was prevented by a inhibitor of protein kinase (staurosporine); PMA partially mimicked the effect of Ang II on the ACTH-induced cAMP response (Baukal et al., 1994). This raises the possibility of an interaction between cAMP and PKC pathways. PKC may be located at a cross-over point of various pathways in hormone signalling action (Nishizuka, 1984). Houslay proposed that PKC might play a pivotal role in modulating relationships between signal transduction pathways (Houslay, 1991). Furthermore, Jacobowitz and Iyengar concluded that the activation of PKC by PMA resulted in phosphorylation and stimulation of AC2, a cell membrane enzyme without binding to G-protein, providing a G-protein-independent mode for signal transduction (Jacobowitz and Iyengar, 1994). In this present work, PMA alone had no effect on cAMP production, consistent with the result obtained by Culty and co-worker (Culty et al., 1984). PMA in combination with ACTH, however,

led to further increases in ACTH-induced cAMP production, implying the influence of PMA on cAMP production might rely on an ACTH trigger.

GF109203X reduced ACTH-stimulated cortisol output but appeared not to alter the levels of total StAR protein, implying that phosphorylation of pre-existing StAR protein by PKC rather than the activation of nuclear transcription might be involved in steroidogenesis, i.e. non-genomic regulation is also important for steroidogenic capacity; or/and other phosphoprotein(s) might participate in steroidogenesis.

Moreover, the stimulatory effects of ACTH on StAR protein and cortisol were also inhibited by KN-93, a specific inhibitor of CaM kinase II, but not KN-92, an analogue of KN-93 in bovine adrenal ZF cells. This is suggestive that the action ACTH is at least partially regulated by a calcium/calmodulin-dependent protein kinase II in addition of PKA and PKC (Nishikawa et al., 1997).

CHAPTER 7 CONCLUDING REMARKS AND FUTURE DIRECTIONS

In the past few decades extensive studies have demonstrated that the availability of cholesterol is an important determinant of steroidogenesis in the adrenal and the gonad. The outstanding fundamental question(s) is (are) what is (are) the regulator (s)/mediator(s) and how does this process happen. Steroidogenic acute regulatory (StAR) protein has been identified to play a crucial role in cholesterol transfer from the outer to the inner mitochondrial membrane where the first step of cholesterol conversion to steroid hormones occurs. This discovery has triggered research on steroidogenesis in many directions at the cellular and molecular levels. The work presented in this thesis has evaluated the role of StAR protein and its relationships to the signalling systems of ACTH-induced steroidogenesis in primary cultures of bovine adrenal ZF cells. The conclusion and speculation raised from this work are summarised as below:

1. Characterisation and validation of a novel anti-bovine peptide StAR polyclonal antibody has permitted detection of StAR protein by Western immunoblotting. Furthermore, a newly formulated protocol based on enhanced chemiluminescence methodology has provided an accurate approach to quantify StAR protein using a molecular image analyser. The linearity and the reliability of this technique have been confirmed by similar testing of an ubiquitous cell protein, actin.

2. The observation of the level of StAR protein expression in the absence and presence of ACTH (10^{-8} M) at separate times over five days of culture allowed the determination of Day 3 as an appropriate starting time for further investigations and treatments. Concentration response curves presented more detailed changes in StAR protein, cortisol and cAMP levels at acute (1 hr) and chronic (6 hr) phases. Marked changes in StAR protein appeared at 6 hr and the greater increases in cortisol output were correlated with greater increases in the levels of StAR protein. At both 1 and 6 hr, the cortisol levels were significantly elevated when the concentrations of ACTH were equal to or greater than 10^{-12} M. While increases in cAMP were measurable at 10^{-11} M and higher concentrations

of ACTH, suggesting cAMP may not be the principal second messenger at low concentrations of ACTH (10^{-12} M) that still elicit increased cortisol secretion.

3. The relationships between StAR protein and cortisol production as well as cAMP formation were studied further through 0.5-24 hr treatment periods. The high concentration ACTH (10^{-8} M) resulted in the temporal appearance of total StAR protein changes coincidental with cortisol production, supporting the notion of a direct relationship between the levels of StAR protein and the steroidogenic capacity under supraphysiological conditions. However, at low concentration ACTH (10^{-12} M), there was a lack of close parallelism between StAR protein and cortisol levels. The difference of the action of high- and low-ACTH was also confirmed by measurements of cAMP production which showed that the changes of cortisol were consistent with those of cAMP at 10^{-8} M ACTH. On the other hand, the cortisol levels were elevated without any notable increase in cAMP over the basal level at 10^{-12} M ACTH, suggesting that the major signalling is possibly cAMP-independent.

4. To identify possible second messengers, in addition to the cAMP, the role of the PLA_2-AA signalling system was evaluated. 4-BPB, a selective inhibitor of PLA_2, impaired the increases in cortisol output induced by ACTH, suggesting that PLA_2-catalysed pathway may be a key approach for releasing AA from phospholipid. Investigation of the downstream AA cascade demonstrated that NDGA, a selective inhibitor of LOX, significantly reduced ACTH-elevated cortisol levels, suggesting that AA-LOX pathway may be major route of AA conversion to its metabolites. INDO, a selective inhibitor of COX, slightly enhanced cortisol output induced by ACTH, implying that the function of separate branches (i.e. COX and LOX) of the AA cascade may differ in mediating steroidogenesis, possibly via a pivotal mechanism. Overall, the effects of these inhibitors were acute and more profound at low concentrations of ACTH (10^{-12} M). Meanwhile, the mediation of cortisol secretion by the third branch of AA metabolism, cytochrome P450-dependent monoxygenase pathway metabolites cannot be ruled out.

5. Results reported herein favour a dual signalling model, cAMP and the PLA_2-AA cascade. The roles of PLA_2-AA signalling pathways are predominantly observed

in the presence of low concentrations of ACTH. The differential activation of second messenger systems may be dependent on the intensity of ACTH treatment. Furthermore, NDGA and INDO that are inhibitors of AA metabolism also affected the cAMP levels, implying an interaction between the two signalling systems. Apart from the cAMP and PLA_2-AA second messenger systems, Ca^{2+} may primarily or/and co-ordinately participate in activation of steroidogenesis; for instance, the involvement of PLA_2 in signal transduction may be dependent on Ca^{2+}. Meanwhile, ACTH-induced increases in cAMP levels may be via a Ca^{2+}-sensitive adenylate cyclase. Ca^{2+} is possibly a cross-over point between cAMP and AA cascades. Therefore, a full spectrum scenario may be that the cAMP pathway, AA cascades and Ca^{2+} are triple second messengers, which constitute signalling networks to mediate steroidogenesis. The signal transduction is not only via the linear correlated multiple factors, but also via the interaction of the factors in a transient or a stable manner. Moreover, cAMP might also play other roles such as in protein-protein interaction in addition to facilitating protein phosphorylation as a conventional second messenger.

6. Both concentration response curves and time courses showed that cortisol was markedly increased acutely without significant alteration in total amount of StAR protein, implying that the initial steroidogenesis may rely on the pre-existing StAR protein, or may be StAR protein-independent. Furthermore, StAR protein did not show notable quantitative variations among inhibitor-untreated and treated cells in the presence of ACTH. This raised the possibility that, in response to, ACTH StAR may change in phosphorylation forms rather than in net protein amount or/and another protein may also be involved in initiating steroidogenesis.

7. PMA, a PKC activator, increased StAR protein without significantly elevating cortisol levels in the absence of calcium ionophore, suggesting that the expression of StAR protein may be regulated by PKC and that Ca^{2+} mobilisation is required for steroidogenesis. Chronically, PMA attenuated ACTH-induced cortisol secretion despite elevated StAR protein levels, implying that an inhibitory effect of PMA on other components (e.g. CYP17) of steroidogenic pathways likely occurred. The inhibitory effect of bisindolylmaleimide I (GF109203X), a highly selective inhibitor of PKC, on cortisol output provided evidence that a

possible sequential activation of steroidogenesis might be PLA_2-AA-PKC. Overall, PKA, PKC and Ca^{2+}/calmudulin-dependent kinase II may be activated through cAMP, the PLA_2-AA and Ca^{2+}signalling pathways respectively which are initially dependent on the concentrations of ACTH.

8. Ang II, an activator of both PKC and Ca^{2+}, moderately enhanced StAR protein and cortisol levels. The effect of the combined Ang II and various concentrations of ACTH on cortisol output exceeded those of Ang II and ACTH alone although the combinations did not produce additive effects on the expression of StAR protein. The mechanism of the interaction of Ang II and ACTH remains unclear.

9. It appears that other factors may also participate in the process of initiating and/or ongoing cholesterol translocation to achieve a flexible and comprehensive steroid output although StAR has been identified as a key mediator in initiating steroidogenesis. PBR and arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt) have emerged as putative candidates in concert with StAR protein to facilitate cholesterol delivery.

Fig. 7-1 illustrates an overview of the main features of this thesis and the possible involvement of other factors.

In summary, there are at least two fundamental mechanisms for steroidogenesis, i.e. the activation of second messenger networks and the regulation of StAR protein, either via rapid non-genomic response (e.g. phosphorylation) or genomic activation (transcription).

In addition, it should be noted that this study was focused on the correlation between the changes of cortisol and StAR protein. However, cortisol is the end product of the steroidogenic pathway in adrenal ZF cells, several components of the steroidogenic pathway also steer the nature steroid synthesis (e.g. CYP11A, CYP17 and CYP11B steroid hydroxylase, etc), since steroidogenesis is a complex and multifactorial process. The co-ordinate roles of those proteins must also be considered, particularly for long term, chronic effects.

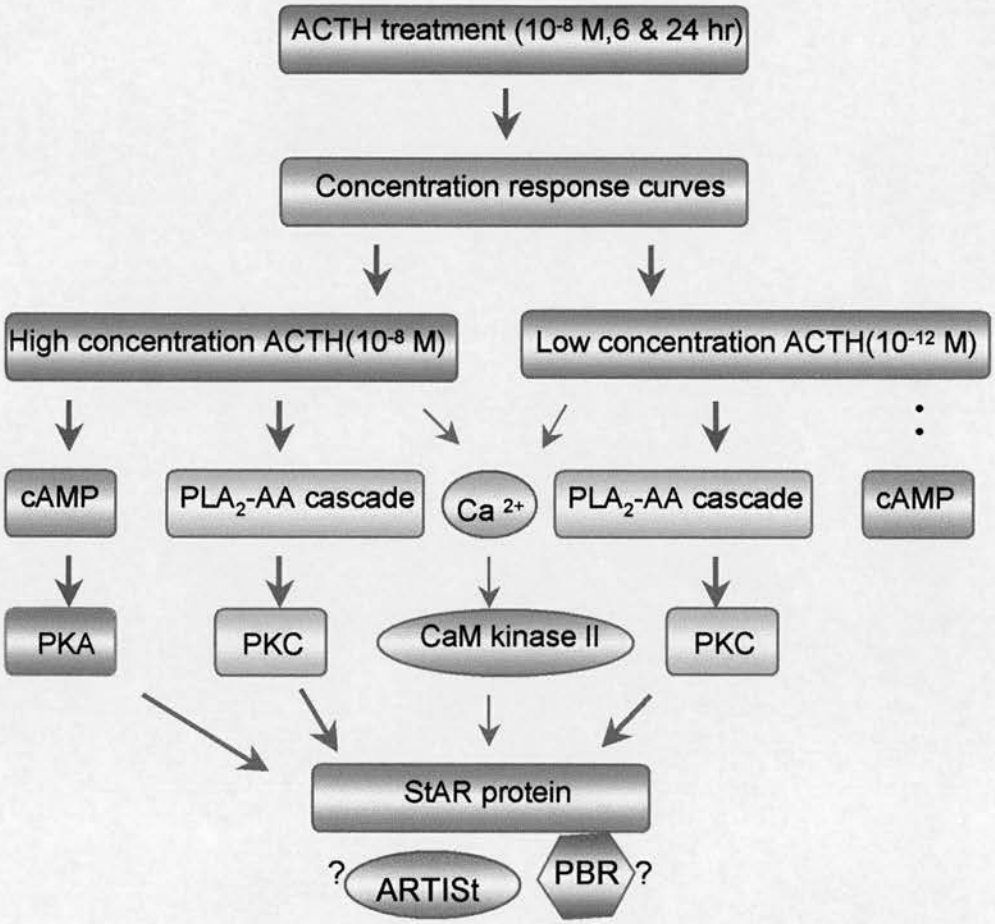

Fig. 7-1 A summary of the main story presented in this thesis and the possible involvement of other factors. PLA_2: Phospholipase A_2; AA: Arachidonic acid; PKA: Protein kinase A; PKC: Protein kinase C; CaM kinase II: Ca^{2+}/calmodulin-dependent kinase II; PBR: Peripheral benzodiazepine receptor; ARTISt: Arachidonic acid-related thioesterase involved in steroidogenesis.

This work should promote future work on several aspects:

1. Phosphorylation of StAR protein in response to hormonal treatment

StAR protein is initially synthesised as a precursor in cytosol and cleaved to mature protein in mitochondria, and can be phosphorylated by several protein kinases. Therefore, StAR protein exists in several forms in terms of molecular mass and isoelectric point value. Because the measurement of total protein amount appears not enough for evaluating the precise action of StAR protein, it would be likely to be of much value to examine the relationship between acute steroidogenesis and various forms of StAR protein in the absence and presence of ACTH. A more detailed investigation would be to look at StAR protein in both mitochondrial-enriched and cytosol fractions. Whether ACTH induces any changes in different forms of StAR protein is now being investigated by 2-D gel electrophoresis. The preliminary data showed that StAR protein diverted to more acidic forms in the presence of 10^{-8} M ACTH.

2. Measurement of pregnenolone

Pregnenolone is the first product of steroidogenic pathways, its content therefore more closely reflects the action of StAR protein/other proteins in facilitating cholesterol transfer. As only low level pregnenolone accumulates in cell culture medium, the measurement probably requires the use of inhibitor(s) for preventing pregnenolone conversion to its products (progesterone and 17-hydroxypregnenolone).

3. Further studies on secondary messengers and protein kinase systems

i) Examination of the role of Ca^{2+} in steroidogenesis and whether Ca^{2+} is a link between the cAMP and PLA_2-AA signalling pathways.

ii) Evaluation of roles for PLA_2-AA signalling pathways using a specific inhibitor. For example, methyl arachidonyl fluorophosphonate (MAFP) is designed as a potent PLA_2 inhibitor *in vitro.*

iii) Investigation of the effect of Ca^{2+}-CaM kinase II on phosphorylation of StAR protein and steroidogenesis.

4. Evaluation of relationships between StAR protein and steroid output using superfusion system

The cortisol levels after hour(s) periods of incubation only reflect accumulated steroid production, whereas the superfusion system is able to demonstrate more accurately cell responses to a treatment within a few seconds/minutes. A precise temporal relationship between StAR protein and steroid output could be determined to understand the acute regulatory mechanism of StAR protein in steroidogenesis.

5. Identification of protein-protein interaction

During the last decade, a number of studies have demonstrated that protein-protein interactions have emerged as a major regulatory mechanism for intracellular networks. One of the sophisticated techniques, yeast two-hybrid system, is designed for elucidating the role of a protein in one or another cellular process (Pirson et al., 1999). The examination of the interactions of StAR-PBR, StAR-ARTISt and PBR-ARTISt may provide a more comprehensive scenario of cholesterol translocation, a key initial step for steroidogenesis.

APPENDIX I MATERIALS OBTAINED FROM COMMERCIAL SOURCES

Amersham Life Science Ltd., Amersham Place, Buckinghamshire HP7 9NA, UK

Cortisol-3-(*O*-carboxymethyl) oximino- (2-[^{125}I] iodohistamine), IM 129

ECL Western blotting analysis system, RPN 2108

BDH-Merck Ltd Hunter Boulevard, Magna Park, Lutterworth LE17 4XN, UK

Acetic anhydride, Prod. 10002

Acetic acid glacial, Prod. 100018Q

Acrylamide, Prod. 44299 4J

Dimethyl sulphoxide, Prod. 103234L

Glycerol, Prod. 10118

NN'-Methylenebisacrylamide, Prod. 44300

Methanol, Prod. 291926G

Sodium acetate anhydrous, Prod. 10236

Sodium dihydrogen orthophosphate, Prod. 30132

Di-sodium hydrogen orthophosphate anhydrous, Prod. 10249

Trisodium citrate, Prod. 102427X

The Binding Site Ltd., P.O. BOX 4073, Birmingham B29 6AT, UK

Donkey anti-sheep/goat immunoglobulins peroxidase, AP 360

Donkey anti-rabbit immunoglobulin peroxidase, AP311

Bio-Rad Laboratories Ltd., Maylands Avenue, Hertfordshire HP2 7TD, UK

30% Acrylamide/Bis solution 37.5:1 (2.6% C), 161-0158

Kaleidoscope pre-stained standards,161-0324

Pre-stained SDS-PAGE standards (low range), 161-0305

Boehringer Mannheim UK (Diagnostics & Biochemicals) Ltd., Bell Lane, Ease Sussex BN7 1LG, UK

Protease inhibitor cocktail tablets, 1697498

Calbiochem-Novabiochem (U.K.) Ltd., Padge Road, Nottingham NG9 2JR, UK

Bisindolylmaleimide I (GF109203X), Cat. No. 203290

Protein kinase C inhibitor 19-27, Cat. No. 476475

Gibco BRL (Life Technologies Ltd.), 3 Fountain Drive, Paisley PA4 9RF, UK

Amphotericin B (250 μg/ml), 15290-026

Dubecco's MEM/NUT mix F-12 (Ham), 31330-038

Earle's balanced salt solution (EBSS), 14015-044

Nutrient mixture Ham's F10, 31550-023

Penicillin/Streptomycin (10,000 units/ml Penicillin and 10,000 μg/ml Streptomycin), 15140-114

Trypsin 2.5%, 25095-019

Lorne Laboratories Ltd., PO Box 6, Twyford, Reading RG10 9NL, UK

Collagenase (Type 1), CLS 1

Marvel Original, Stafford ST20 0OJ, UK

Dried skimmed milk powder (99% fat free)

National Institute for Biological Standards and Control (NIBSC), Potters Bar, Herts, UK

Angiotensin II (asp^{1}-val^{5}, WHO standard 64/15)

Novartis Pharma AG, CH-4332, Stein, Switzerland

Synacthen Ampoules ($ACTH_{1-24}$)

Pierce & Warriner (UK) Ltd., 44 Upper Northgate Street, Chester CH1 4EF, UK

SuperBlock blocking buffer in PBS, 37515

SuperSignal West dura extended duration substrate, 34075

Santa Cruz Biotechnology, Inc., California 95060, USA

Actin (1-19), sc-1616

Scottish Antibody Production Unit (SAPU), Lanarkshine, ML8 5ES, UK

Donkey anti-rabbit serum, S 022-220

Donkey anti-sheep/goat IgG, S024-220

Normal rabbit serum, S119-205

Normal sheep serum, S031-220

Sheep anti-cortisol serum, S004-201

Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH, UK

Arachidonic acid, A-3555

Albumin, Bovine (Fraction V, Fatty acid free), A-8806

Albumin, Bovine (Fraction V), A-9418

Ammonium persulfate, A-3678

Brilliant blue G (Coomassie brilliant blue G-250), B-0770

4-Bromophenacyl bromide, B-2006

Controlled process serum replacement-1, C-8905

Deoxycholic acid (sodium salt), D-6750

Gelatin (Type B), G-6269

Hydrocortisone, H-4001

Indomethacin, I-7378

Kodak X-OMAT film, F-5513

Kodak X-OMT LS film, F-1149

Lauryl sulfate (SDS, sodium dodecylsulfate), L-3771

Immobilon-P polyvinylidene difluoride (PVDF), P-2938

2-Mercaptoethanol, M-7154

Nordihydroguaiaretic acid, N-5023

Polyxyethylene sorbitan monolaurate (Tween 20), P-1379

Phorbol-12-myristate 13-acetate, P-8139

Sephadex G-10, G-10-120

Sephadex G-50, G-50-150

Sucrose, S-9378

N,N,N',N'-Tetramethylethylenediamine, T-9281

Triethylamine, T-0886

Tris (hydroxymethyl) aminomethane, T-6791

APPENDIX II REFERENCES

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APPENDIX III PUBLICATIONS AND POSTER PRESENTATIONS ARISING FROM THIS THESIS WORK

PUBLICATIONS

Wang H, Walker SW, Mason JI, Morley SD, Williams BC. (2000) Role of arachidonic acid metabolism in acute ACTH-stimulated cortisol secretion by bovine adrenal zona fasciculata (ZF) cells. *Endocrine Res.* **26** (4):705-709.

Ivell R, Tillmann G, **Wang H,** Nicol M, Stewart PM, Bartlick B, Walther N, Mason JI, Morley SD. (2000) Acute regulation of the bovine gene for the steroidogenic acute regulatory protein in ovarian theca and adrenocortical cells. *J. Mol. Endocrinol.* **24**: 109-118.

Nicol M, **Wang H**, Ivell R, Morley SD, Walker SW, Mason JI. (1998) The expression of Steroidogenic Acute Regulatory protein (StAR) in bovine adrenocortical cells. *Endocrine Res.* **24** (3&4): 565-569.

POSTER PRESENTATIONS

Wang H, Walker SW, Mason JI, Morley SD, Williams BC. 2000 Role of arachidonic acid metabolism in acute ACTH-stimulated cortisol secretion by bovine adrenal zona fasciculata (ZF) cells. IXth Adrenal Cortex Conference, June 2000, Toronto, Canada.

Wang H, Nicol M, Walker SW, Mason JI, Morley SD, Williams BC. (1999) Regulation of StAR protein during high & low concentration ACTH-stimulated steroidogenesis in primary bovine adrenal zona fasciculata cells. J. Endocrinol. vol. 163 Abstract Suppl. P95. Society for Endocrinology, 190th Meeting, November 1999, London, UK.

Ivell R, Tillmann G, **Wang H,** Nicol M, Stewart PM, Bartlick B, Walther N, Mason JI, Morley SD. (1999) Acute regulation of the bovine gene for the steroidogenic acute regulatory (StAR) protein in ovarian theca and adrenocortical cells. J. Endocrinol. vol. 163 Abstract Suppl. P98. Society for Endocrinology, 190th Meeting, November 1999, London, UK.

Nicol M, **Wang H**, Ivell R, Morley SD, Walker SW, Mason JI. (1998) The expression of Steroidogenic Acute Regulatory protein (StAR) in bovine adrenocortical cells. VIIIth Adrenal Cortex Conference, June 1998, Sherbrooke, Quebec, Canada.

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ROLE OF ARACHIDONIC ACID METABOLISM IN ACTH-STIMULATED CORTISOL SECRETION BY BOVINE ADRENOCORTICAL CELLS

H. Wang, S.W. Walker, J.I. Mason, S.D. Morley and B.C. Williams*
Department of Reproductive & Developmental Sciences (Clinical Biochemistry), University of Edinburgh, Royal Infirmary, Edinburgh EH3 9YW; *Department of Medical Sciences, Western General Hospital, Edinburgh EH4 2XU, UK.

ABSTRACT

We have studied the effects of inhibitors of arachidonic acid (AA) metabolism, nordihydroguaiaretic acid (NDGA), a lipoxygenase (LPX) inhibitor, and indomethacin (INDO), a cyclooxygenase (COX) inhibitor, on cortisol secretion and StAR protein in primary cultures of bovine adrenal zona fasciculata (ZF) cells. NDGA inhibited cortisol secretion in response to both 10^{-12} M and 10^{-8} M ACTH. AA (10^{-4} M) partially reversed the inhibition of cortisol secretion by NDGA at 10^{-12} M ACTH but not at 10^{-8} M ACTH. On the other hand, INDO potentiated the cortisol response to 10^{-12} M ACTH. Neither NDGA nor INDO significantly affected StAR protein levels. These results suggest a StAR protein-independent role for the LPX and COX pathways in acute cortisol secretion, and support the hypothesis that LPX products of AA metabolism are key cellular signals when bovine ZF cells are acutely stimulated by physiological concentrations of ACTH (10^{-12} M).

INTRODUCTION

Our previous results have demonstrated that the secretion and expression profiles for cortisol and Steroidogenic Acute Regulatory (StAR) protein differ in response to high (10^{-8} M) versus low (10^{-12} M) ACTH treatment of bovine ZF cells (1). There was a strong positive correlation between cortisol secretion and

StAR protein induction in response to 10^{-8} M ACTH but not 10^{-12} M ACTH. The intracellular mechanism for ACTH-stimulated steroidogenesis at high concentrations is principally via a cAMP dependent pathway (2-4). On the other hand, several studies strongly suggest that arachidonic acid (AA) metabolites may be important second messengers for ACTH stimulation (5, 6). The purpose of this study was to investigate the role of the AA pathway in the stimulatory effects of ACTH, at both high (10^{-8} M) and low (10^{-12} M) concentrations, on cortisol output and StAR protein levels.

MATERIALS AND METHODS

Nutrient mixture Ham's F10 medium and antibiotics were obtained from Life Technologies Ltd (Paisley, UK). $ACTH_{1-24}$ was purchased from Alliance Pharmaceutical Ltd (Chippenham, Wiltshire, UK). CPSR-1, bovine serum albumin (BSA), arachidonic acid, nordihydroguaiaretic acid and indomethacin were obtained from Sigma (Poole, Dorset, UK). After isolation, bovine ZF (1.5 $x10^6$/35 mm well) cells were cultured in Ham's F10 containing 10% (v/v) CPSR-1 for 48 hr prior to the medium being replaced overnight with Ham's F10 containing 0.2% (w/v) BSA (7, 8). Cells were treated with ACTH (10^{-12} M or 10^{-8} M) after 15 min pre-incubation with NDGA or INDO. Cortisol in the medium was measured by radioimmunoassay. StAR protein levels were quantified by Phosphor Imaging analysis of ECL western immunoblots (8). Statistical analyses were performed with ANOVA, followed by Student-Newman-Keuls multiple comparison test.

RESULTS AND DISCUSSION

As shown in Fig 1A, $5x10^{-5}$ M NDGA inhibited 10^{-12} M- and 10^{-8} M ACTH-stimulated cortisol production and $2x10^{-6}$ M NDGA also decreased 10^{-12} M ACTH-stimulated cortisol secretion but not that at 10^{-8} M ACTH, suggesting that NDGA was more inhibitory at the lower ACTH concentration. The addition of 10^{-5} M or 10^{-4} M INDO increased 10^{-12} M ACTH-stimulated cortisol secretion at

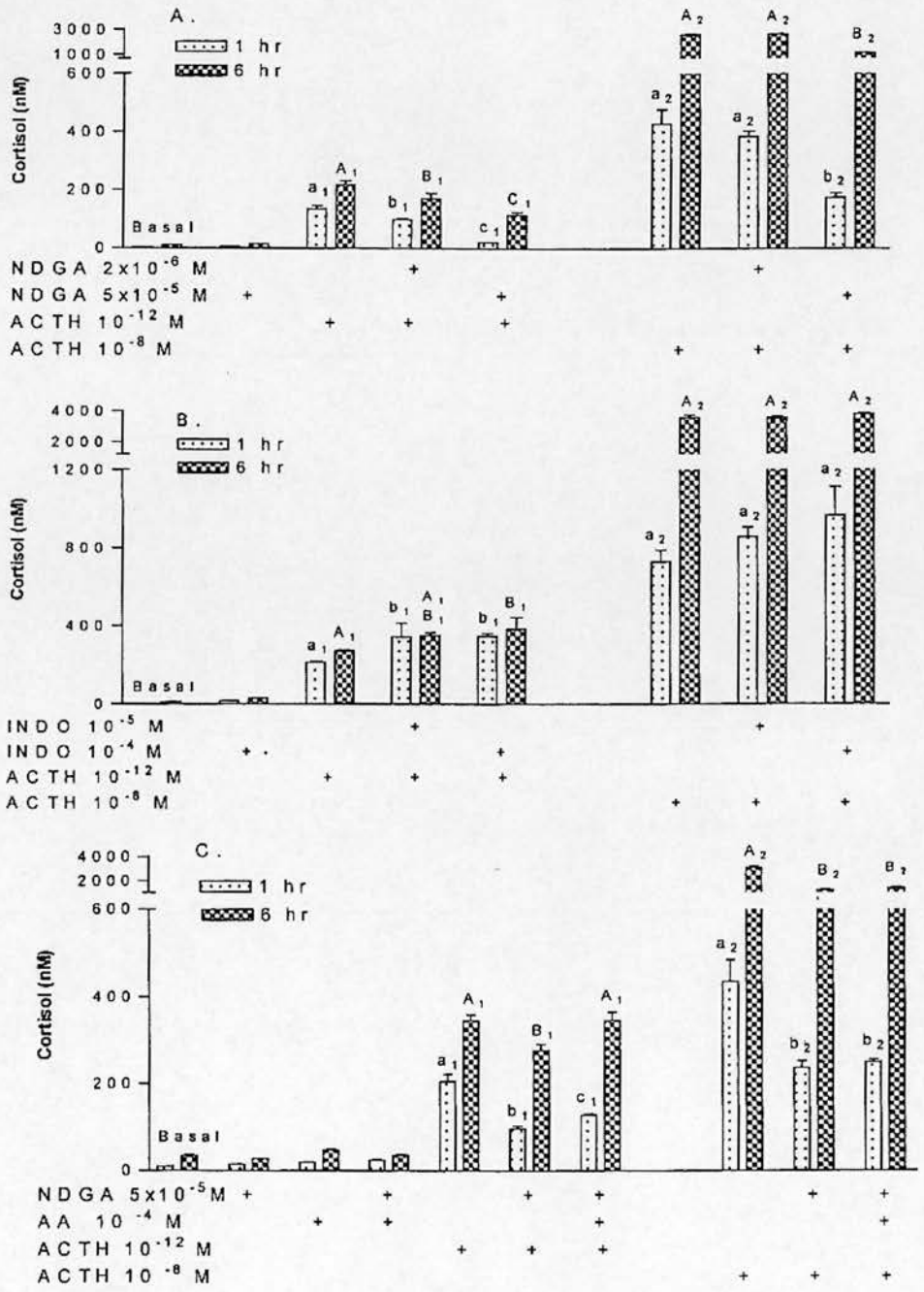

FIGURE 1

Effects of NDGA (A), INDO (B) and NDGA plus arachidonic acid (C) on basal and ACTH-promoted cortisol production. Values are expressed as mean ± SE (n=3); groups without the same superscript letters are significantly different ($p<0.05$ or <0.01). Letters in lower and upper case indicate 1 and 6 hr time points, respectively. Subscripts "1" and "2" represent 10^{-12} M and 10^{-8} M ACTH treatments, respectively.

1 hr and 10^{-4} M INDO enhanced 10^{-12} M ACTH-stimulated cortisol secretion at 6 hr. Neither concentration of INDO significantly affected 10^{-8} M ACTH-stimulated cortisol output (Fig 1B). In all treatments with inhibitors, there were no measurable alterations in ACTH-induced StAR protein levels (data not shown). The addition of arachidonic acid rescued the inhibitory effect of NDGA on 10^{-12} M ACTH-stimulated cortisol output at both 1 and 6 hrs. But the cortisol secretion was less effectively restored at 1 hr, indicating that NDGA has a more marked effect on cortisol production at the early time point. AA only marginally changed the NDGA-inhibited cortisol output at 10^{-8} M ACTH (Fig 1C).

AA is released from membrane phospholipids by the action of phospholipase A_2 (9) and then can be further metabolised to other eicosanoids via two main pathways (LPX and COX). AA and its metabolites have been demonstrated to function as second messengers in response to trophic hormone stimulation (10-12). Our results have shown that the inhibition of NDGA was more notable at 10^{-12} M ACTH and in the acute phase of steroidogenesis. The enhanced effect of INDO on 10^{-12} M ACTH-stimulated cortisol production suggests that the metabolism of AA through cyclooxygenase may be prevented and redirected into lipoxygenase metabolites, thereby leading to increased cortisol secretion.

In conclusion, (i) arachidonic acid and its metabolites may be a key second messenger system for steroidogenesis in response to low concentrations of ACTH (10^{-12} M); (ii) high concentrations of ACTH (10^{-8} M) which stimulate the cAMP signalling pathway may also activate the arachidonic acid cascade; (iii) StAR protein levels remain unchanged after treatment with AA pathway inhibitors. This raises the possibility that the role of AA may be independent of newly synthesised StAR protein; or that StAR protein may co-operate with other proteins (e.g. ARTISt, PBR) in regulating cortisol secretion in bovine adrenal ZF cells.

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Acute regulation of the bovine gene for the steroidogenic acute regulatory protein in ovarian theca and adrenocortical cells

R Ivell[1], G Tillmann[1], H Wang[2], M Nicol[2], P M Stewart[3], B Bartlick[1], N Walther[1], J I Mason[2] and **S D Morley[2]**

[1]Institute for Hormone and Fertility Research, University of Hamburg, Grandweg 64, 22529 Hamburg, Germany
[2]Department of Reproductive and Developmental Sciences (Clinical Biochemistry section), University of Edinburgh, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh EH3 9YW, UK
[3]Department of Medicine, University of Birmingham Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TH, UK

(Requests for offprints should be addressed to R Ivell; Email: ivell@ihf.de)

ABSTRACT

Upregulation of the steroidogenic acute regulatory protein (StAR) is implicated in the rapid synthesis and secretion of steroidogenic cells to produce steroids in response to stimulation by trophic hormones of the gonadal and stress axes. In the present study, we have assessed the kinetics of both *StAR* gene transcription and protein biosynthesis in primary cell cultures of bovine adrenocortical and ovarian theca cells, under conditions of acute stimulation by corticotrophin (ACTH) and luteinizing hormone (LH), respectively. In both cell systems, detectable upregulation of *StAR* gene transcription occurred within 1–2 h, reaching maxima at 4 h (theca cells) or 6 h (adrenocortical cells). mRNA levels returned rapidly to baseline, by 12 h or 24 h, respectively. Specific StAR protein levels were assessed by western blotting using a novel antibody raised against a bovine StAR peptide, and showed a similar fast upregulation, albeit delayed by 1–2 h compared with the mRNA. The response of the cultured theca cells was more acute than that of the adrenocortical cells, possibly reflecting the propensity of the LH receptor to desensitize rapidly, unlike the ACTH receptor. The primary bovine theca cell cultures were also used for fully homologous transfection studies using various deletion promoter–reporter constructs of the bovine *StAR* gene. Kinetic analysis of the results indicated that the acute transcriptional response resides within the proximal (− 315 bp) promoter region, which includes two putative responsive elements for the steroidogenic factor-1. More distal promoter regions may be involved in modulating the specificity of expression by combining enhancer and inhibitory functions.

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INTRODUCTION

In steroidogenic tissues, the rate-limiting step in the acute hormone-dependent upregulation of steroid biosynthesis is considered to be the transport of the cholesterol substrate to the inner mitochondrial membrane, where it is converted to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage complex (reviewed in Thomson 1998). This rate-limiting mitochondrial transport is dependent upon the *de novo* biosynthesis of new protein (reviewed in Stocco & Clark 1996). An important candidate for this role is the recently cloned steroidogenic acute regulatory protein (StAR; Clark *et al.* 1994), which is rapidly and highly upregulated after a steroidogenic stimulus, and becomes associated with mitochondria (Stocco & Clark 1996). Furthermore, the apparently indispensible nature of StAR is revealed by the severe impairment of steroidogenesis after inactivation of the *StAR* gene in mice by homologous recombination (Caron *et al.* 1997*b*), or in the naturally occurring human deficiency disease, congenital lipoid adrenal hyperplasia (Miller 1997).

The cDNA and gene sequence encoding the StAR protein have been elaborated, initially for the mouse (Clark *et al.* 1994), and subsequently for other species, including the bovine (Hartung *et al.* 1995). Initial experiments investigated the basal levels of StAR mRNA in different tissues *in vivo*, or in tumour cell lines, and the levels attained after long-term stimulation. Although several studies have looked at factors regulating the *StAR* gene promoter (reviewed in Reinhardt *et al.* 1999*b*), most of these used a prolonged (>12 h) stimulation protocol, and so the molecular mechanisms responsible for the acute (<4 h) cAMP-dependent upregulation of *StAR* gene transcription remain unclear. In order to investigate this in a homologous system, we have exploited the ability of bovine ovarian theca cells and bovine adrenocortical cells to produce large amounts of steroids in response to acute stimulation by the natural adenylate cyclase-linked secretagogues, luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH), respectively. Using northern and western blotting for StAR mRNA and protein, we first characterized the time-course of the acute specific stimulation of *StAR* gene transcription in these cell types by their natural effectors. Subsequently, we used transfection of previously characterized promoter–deletion–reporter constructs derived from the bovine *StAR* gene (Rust *et al.* 1998) into cultured bovine primary ovarian theca cells to provide a completely homologous system in which to delineate elements of the StAR promoter that are required for the rapid LH-dependent increase in transcription. These results show that the time- and hormone-specificity of *StAR* gene transcription reside within the proximal promoter region, between − 315 and the transcriptional start site.

MATERIALS AND METHODS

Cell culture

Bovine theca interna cells were prepared from large antral follicles (10–25 mm diameter) of ovaries collected from mid- to late-cycle cows at the local abbatoir. Cell preparation and primary culture conditions were exactly as described by Bathgate *et al.* (1999). After Percoll purification, cells were resuspended in 1 : 1 Dulbeccos's minimal essential medium and Ham's F-12 medium, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0·1% BSA, 5 μg/ml transferrin, and 5 ng/ml sodium selinite. For mRNA and protein preparation, approximately 10^6 cells were seeded into vitrogen-coated six-well plates and cultured at 37 °C under 95% air–5% CO_2 for 6 days (with medium changed every 2 days), attaining in that time approximately 80% confluence. For measurements of endogenous mRNA and protein production, after the medium change on day 6, cells were supplemented in addition with 100 ng/ml bovine insulin with or without 10 ng/ml bovine luteinizing hormone (bLH; a kind gift of NIADDK and the National Hormone and Pituitary Program, NIH, Bethesda, MD, USA), and culture continued for the times indicated. Primary zona fasciculata/reticularis (ZFR) cells were isolated from sliced bovine adrenocortical tissue as previously described (Williams *et al.* 1989, Nicol *et al.* 1998) and were plated at a density of $1{\cdot}5 \times 10^6$ per well of a six-well plate for both mRNA and protein studies. After plating, cells were maintained for 48 h in Ham's F-10 medium containing 10% v/v controlled processed serum replacement-1 (Sigma, Poole, Dorset, UK), 100 IU penicillin, 100 μg/ml streptomycin and 5 μg/ml amphotericin B at 37 °C under 95% air–5% CO_2, with one medium change after 24 h, and then for a further 16 h in Ham's F-10 medium containing 0·2% BSA and antibiotics as above, before hormone treatment using 10 nM Synacthen (synthetic ACTH(1–24) peptide, Novartis Pharmaceuticals, Camberley, Surrey, UK) in the same medium.

Northern hybridization

For the ovarian theca cells, total RNA was extracted using the peqGOLD RNA-Pure (peqLab, Erlangen, Germany) reagent and pooled from two wells per experiment for each data point. Two micrograms total RNA per slot were subjected to northern hybridization using 1·3% agarose–2·2 M formaldehyde gels in morpholinopropanesulphonic acid running buffer (Sambrook *et al.* 1989). RNA was transferred to a nylon membrane (Nytran; Schleicher & Schüll, Dassell, Germany) by overnight capillary transfer and fixed by u.v. crosslinking. Hybridization with a 750 bp gene-specific probe from the 5′ region of the bovine StAR cDNA was exactly as previously described (Hartung *et al.* 1995). To control for even loading and transfer of the RNA, blots were rehybridized using a radiolabelled probe specific for the bovine S15 ribosomal protein (Bathgate *et al.* 1999). For the ZFR cells, total RNA was isolated using the RNAzol B reagent (Biogenesis, Poole, Dorset, UK), and 20 μg RNA per slot was denatured with glyoxal (Thomas 1983), resolved on a 1·2% agarose gel in 10 mM sodium phosphate pH 7·0 buffer, capillary-transferred to positively charged nylon membranes (Stratagene) and fixed by u.v. crosslinking. Hybridization for StAR mRNA was as above, but using a somewhat

shorter 5′ cDNA probe (accession number S79908, nucleotides 210–608). Even loading and transfer were confirmed by rehybridization using a β-actin probe. All northern and western (see below) blotting experiments were repeated at least twice for fully independent batches of cells, and gave fully reproducible results.

Antibody production and western blotting

Total cellular protein (ZFR cells 25 μg, theca cells 15 μg per sample), was prepared by homogenization in PBS containing 0·1% SDS and 1% sodium deoxycholate, resolved on a 12% acrylamide gel, and electroblotted onto Immobilon-P membrane (Sigma). Western analysis was carried out using, as primary antibody, a polyclonal sheep antiserum raised against a peptide fragment (AMQRALGIL KDQEGWKKESRQANGDE; amino acids 82–107; Hartung *et al.* 1995) from the predicted bovine StAR protein sequence attached to a lysine-web-based eight-branched antigen scaffold (The Binding Site, Birmingham, UK), and a donkey anti-sheep horseradish peroxidase-conjugated second antibody. After preliminary optimization (data not shown), primary and secondary antibodies were used respectively at 1 : 10 000 and 1 : 25 000 dilutions in PBS containing 10% blocking buffer (Pierce, Rockford, IL, Staffs, USA) and 2% w/v non-fat milk (Marvel Original; Premier Beverages, Stafford, UK) for immunodetection of adrenocortical proteins, and at 1 : 7500 and 1 : 10 000 dilutions, respectively, in PBS containing 10% blocking buffer (Pierce) and 1% non-fat milk for theca cell proteins. The final signal was visualized by chemiluminescence (SuperSignal ULTRA kit; Pierce). The specificity of the antibody was demonstrated first by its ability to detect a protein in adrenocortical protein extracts at the anticipated size of approximately 30 kDa, the pattern of expression of which followed closely behind that of the StAR mRNA (see later), and, secondly, by the complete elimination of the specific 30 kDa band as a result of the addition of an excess (>3 ng/ml) of the peptide used to generate the antibody (not shown).

Cell transfection and analysis

All bovine StAR promoter–reporter DNA constructs, in addition to control vectors, were exactly as previously described (Rust *et al.* 1998). DNA was purified using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) as described by the manufacturer. For transfection experiments, 10^6 bovine theca cells per well, prepared as above, were seeded into 12-well plates, and cultured as above in the presence of 100 ng/ml insulin for 2 days. Cells were then washed briefly in PBS before the addition of OPTI-MEM (Gibco-BRL, Deisenhofen, Germany) transfection medium. Transfection was carried out by adding 5 μg per well LipofectAMINE (Gibco-BRL) at a ratio of 2 : 1 with the DNA to be transfected, and incubating for 5 h. Medium was then replaced by the standard culture medium and incubation continued for a further 3 days, before treatment or not with 10 ng/ml bLH, for the times as indicated. Cells were then washed rapidly in PBS and immediately extracted into 40 μl reporter lysis buffer (Luciferase Assay System; Promega, Madison, WI, USA) before luciferase activity was measured using the same kit. As cotransfected control, a β-galactosidase reporter gene driven from the cytomegalovirus (CMV) promoter was used, measuring the resulting activity using the Galacto-Light kit (Tropix, Bedford, MA, USA). All transfections were performed in triplicate for any batch of primary cells. All experiments were repeated at least twice using independent batches of cells, with fully reproducible results.

RESULTS

Expression of endogenous StAR mRNA and protein in primary cultures of bovine ovarian theca and adrenocortical (ZFR) cells

The endogenous StAR mRNA in bovine ovarian theca and adrenocortical (ZFR) cells was expressed as transcripts of three different sizes (Figs 1, 2), as shown previously also for bovine corpus luteum (Hartung *et al.* 1995, Pescador *et al.* 1996) and predicted from the positions of alternative polyadenylation sites within the 3′ UTR of the cloned bovine cDNA (Hartung *et al.* 1995). These migrated as two major bands at approximately 3·0 kb and 1·8 kb, and a minor band at 1·6 kb, evident only in the theca cells. Levels in untreated cells were very low for both cell types (Figs 1B, 2B). After treatment of theca cells with bLH or adrenocortical cells with ACTH, there was a similar rapid increase in the relative amounts of the two major StAR transcripts in both cell systems. This increase was already detectable after 1 h of stimulation, by comparison with the untreated controls, and reached a maximum at about 4 h (theca cells, Fig. 1A) and 6 h (adrenocortical cells, Fig. 2A) of treatment. At the time of maximal induction of StAR mRNA, the shorter 1·6 kb transcript became readily detectable in the theca cell cultures (Fig. 1A). Also of significance was the rapid decline in transcript levels at subsequent

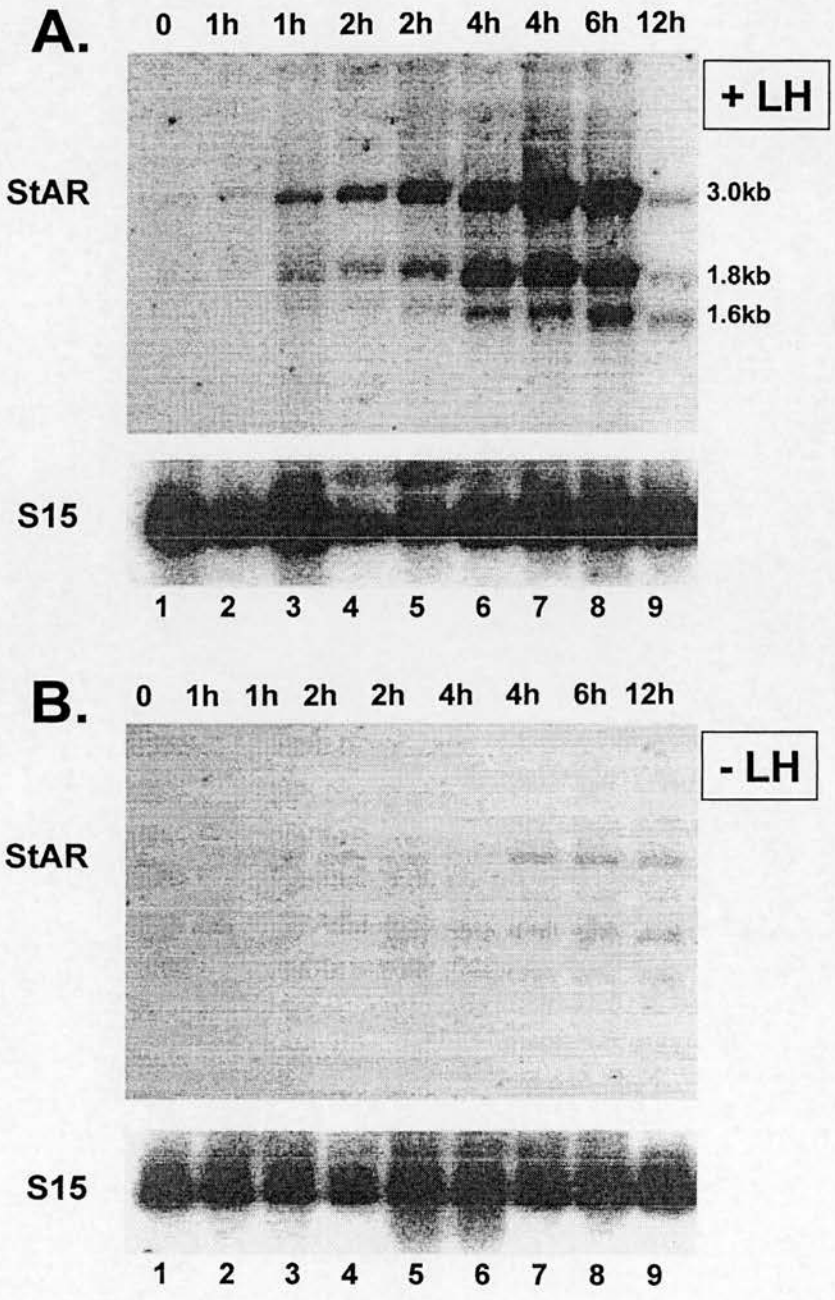

FIGURE 1. Northern hybridization of StAR mRNA in extracts of bovine theca cells cultured for the time indicated in the presence (A) or absence (B) of 10 ng/ml LH. As control for even loading and transfer, blots were rehybridized against a probe for the bovine ribosomal protein, S15. The blots in (A) and (B) are from parallel experiments using the same batch of cells. Duplicate lanes represent RNA from independent parallel cell cultures from within the same experiment, to indicate the extent of within-batch variation.

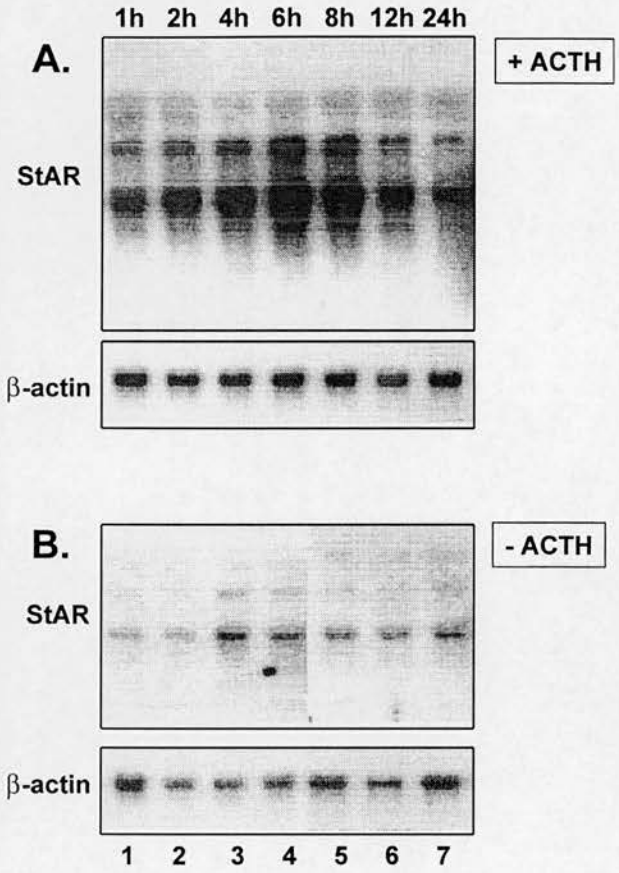

FIGURE 2. Northern hybridization of StAR mRNA in extracts of bovine adrenocortical (zonae fasciculatis and reticularis) cells cultured for the times indicated in the presence (A) or absence (B) of 10 nM ACTH. As control for even loading and transfer, blots were rehybridized against a probe for β-actin. The blots in (A) and (B) are from parallel experiments using the same batch of cells, with all operations being performed in parallel.

time-points, such that, at 12 h (theca cells) or 24 h (adrenocortical cells), StAR mRNA had returned to basal (unstimulated) levels. The apparent and slight long-term increase in StAR mRNA in the absence of bLH (Fig. 1B) probably reflected the differentiation of these cells in culture under the influence of insulin only (Bathgate *et al.* 1999).

Total cellular protein was also prepared from similar cultures of stimulated and unstimulated cells and subjected to western blotting using a newly developed anti-StAR polyclonal antibody. This antibody was raised against a 26-mer sequence from the predicted external surface of the bovine StAR protein (Hartung *et al.* 1995) and specifically recognized a 30 kDa protein (Fig. 3). Protein bands of similar size were detected in adrenocortical and luteal protein extracts using another polyclonal antibody raised against mouse recombinant StAR protein (Pescador *et al.* 1996, Ronen-Fuhrmann *et al.* 1998, and data not shown). The time-course of changes in StAR protein levels in the two cell types was similar (Figs 3, 4), with a first detectable increase at 2 h and maximum levels being maintained through to 6 h (theca cells) or somewhat longer (adrenocortical cells), but with a return to near basal values by 12–24 h. Thus StAR protein production was marginally delayed, both in appearance and disappearance, with respect to the changes in levels of the specific mRNA. Absolute levels of StAR protein, both basal and stimulated, appear to be much greater in the adrenocortical cells than in the bovine theca cells, as indicated by the control lanes (1 and 2) in Fig. 3, in which the same amount of total protein as in the other lanes had been loaded.

Acute regulation of the bovine StAR gene promoter in homologous cell culture

In a previous study (Rust *et al.* 1998), we have been able to characterize the role of certain *cis* elements within the upstream promoter region of the bovine *StAR* gene responsible for expression in a heterologous cell system cotransfected or not with the transcription factor, steroidogenic factor-1 (SF-1; Ad4 BP; Fig. 5). In this system, deletion promoter–reporter constructs had been transfected into non-steroidogenic cells together with appropriate expression vectors. In order to determine whether the SF-1-responsive elements are also involved in endogenous *StAR* gene expression in primary cultures of bovine steroidogenic cells (i.e. in a homologous system as close to the *in vivo* situation as possible), and are sufficient to account for the acute up- and downregulation of the *StAR* gene, a time-course for activation of defined promoter–reporter constructs was determined under acute bLH stimulation after transfection into primary ovarian theca cells (Fig. 6). The results show that there was consistently detectable upregulation in response to bLH by 3 h of stimulation. The maximum level of luciferase activity accumulated in the cells was reached by 4–6 h, and was not further increased at later times. A comparable time-course was obtained irrespective of the use of a long promoter fragment (−1245 bp; Fig. 6A), or a shorter fragment (−315 bp; Fig. 6B) attached to the luciferase reporter gene. However, it should be noted that, in repeated experiments, the response to LH appeared generally to be more robust, consistent, and somewhat earlier in these primary cell

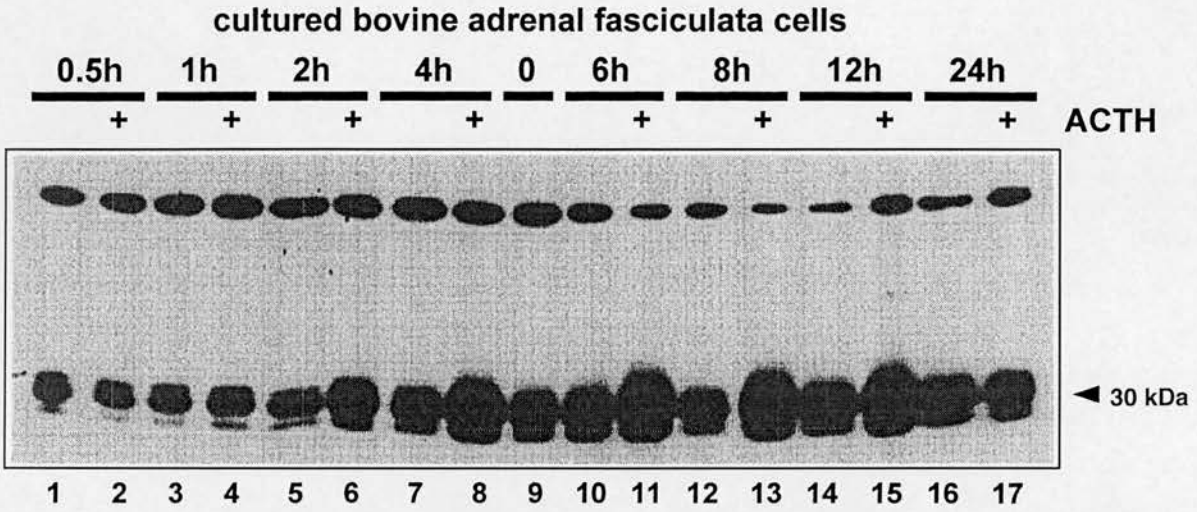

FIGURE 3. Western blot of immunoreactive StAR protein in extracts of bovine adrenocortical cells cultured as indicated for increasing times in the absence or presence (+) of 10 nM ACTH. The upper, more slowly migrating band appears to be non-specific and its intensity does not alter with time or incubation conditions. The specific, approximately 30 kDa StAR protein appears as a doublet with a more rapidly moving isoform increasing in intensity, in parallel with the principal StAR protein band. In all lanes, 25 μg protein were loaded.

cultures, for the shorter −315 bp construct (cf. Fig. 7).

Experiments using different promoter–deletion constructs transfected into theca cells optimally stimulated by bLH for 4 h indicated that the acute stimulation of the bovine *StAR* gene requires only the minimal promoter up to −315 bp (Fig. 7), which includes the first two proximal SF-1 binding motifs (Rust *et al.* 1998). Indeed, even shorter constructs comprising only one (− 203) or none (− 101) of the proximal SF-1 sites, also appear to be responsive to LH. Longer promoter constructs, however, do not increase the level of luciferase activity obtained. As already indicated in Fig. 7, these appear to have reduced activity by comparison with the shorter constructs. This would suggest that the region upstream of the −315 construct may include a negative factor that could modulate the activity of the proximal promoter.

DISCUSSION

The acute response of steroidogenic cells to the anterior pituitary hormones, LH or ACTH, is an essential part of the regulatory mechanisms promoting steroid biosynthesis. Both peptide hormones are released in short pulses or trains of pulses, and elicit both immediate and sustained effects. Amongst the immediate effects are those influencing steroid secretion (reviewed in Thomson 1998). In addition to such very acute events, there is also an effect at the transcriptional level. This effect was quite rapid, newly transcribed StAR mRNA being detectable in both adrenocortical and ovarian thecal cells after about 1 h of stimulation. Equally important, however, is the observation, also for both cell types, that levels of mRNA peaked at about 4–6 h and then declined to basal values by, maximally, 12–24 h. This shows that the stimulatory effect upon transcription is short-lived, and that mechanisms are present in these steroidogenic cells for the rapid degradation of the newly synthesized StAR mRNA. It is important to note that the primary cell cultures were exposed continuously, through the treatment period, to the effectors LH or ACTH. It is known that the LH receptor is rapidly downregulated (desensitized, internalized) within the first few minutes of hormone exposure (e.g. for bovine luteal cells; Budnik & Mukhopadhyay 1987), thus all subsequent LH-dependent events will be receptor-independent consequences of this initial stimulation. For the effect of ACTH on adrenocortical cells, the situation appears to be different. Instead of being desensitized, the cognate receptor appears rather to be upregulated (Penhoat *et al.* 1989). This could explain the more sustained transcriptional response of the *StAR* gene to ACTH treatment evident in the adrenocortical cell cultures.

By transfecting different promoter–reporter constructs into ovarian theca cells, under a treatment paradigm similar to that above in which we have

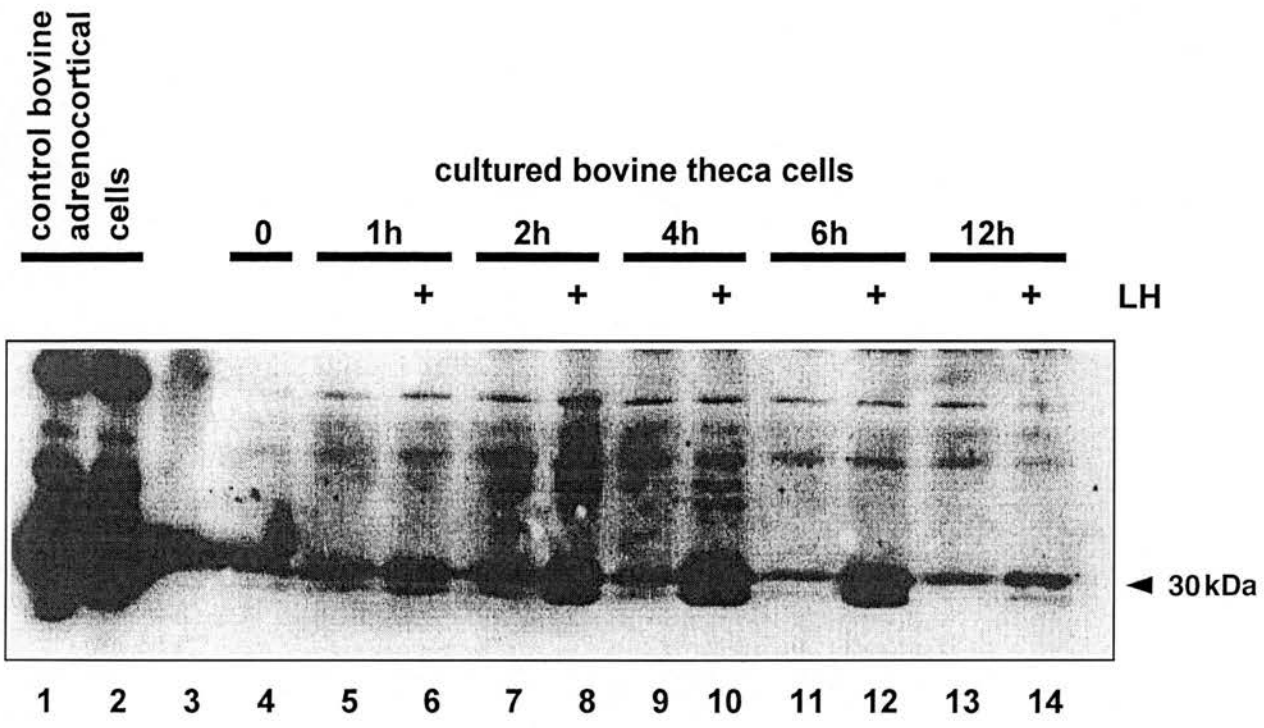

FIGURE 4. Western blot of immunoreactive StAR protein in extracts of bovine theca cells cultured as indicated for increasing times in the absence or presence (+) of 10 ng/ml bovine LH. It should be noted that the absolute levels of the StAR protein are considerably less than in the adrenocortical cells (lanes 1 and 2), and there is thus an increased non-specific background. As for the adrenocortical cells, the StAR protein appears to be represented as a doublet. In all lanes, 15 μg protein were loaded.

shown acute upregulation of the endogenous *StAR* gene, we were also able to show that luciferase activity (i.e. expression of active protein) follows the same time-course of induction as the native StAR protein. Increased luciferase activity was evident first at 2–3 h, just like the endogenous StAR protein seen in the western blots, suggesting that both StAR and luciferase mRNAs are translated in a comparable manner. The induction of reporter activity was, as expected, slightly delayed by comparison with the induction of transcription. The disappearance of the luciferase activity will probably have a

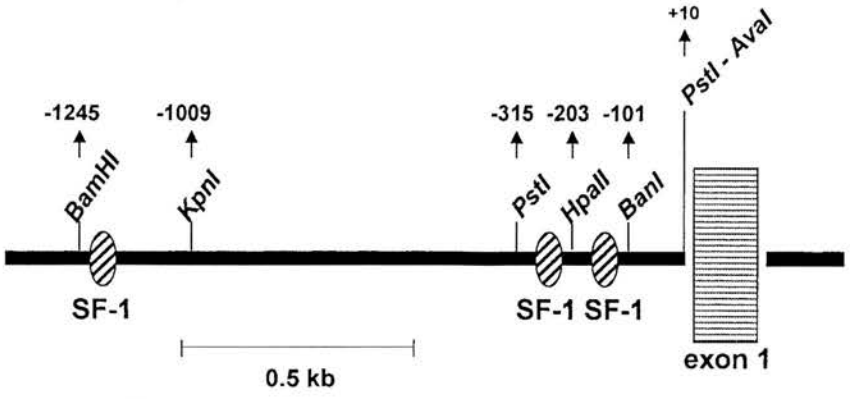

FIGURE 5. Schematic representation of the bovine *StAR* gene and its promoter, indicating putative transcription factor binding elements.

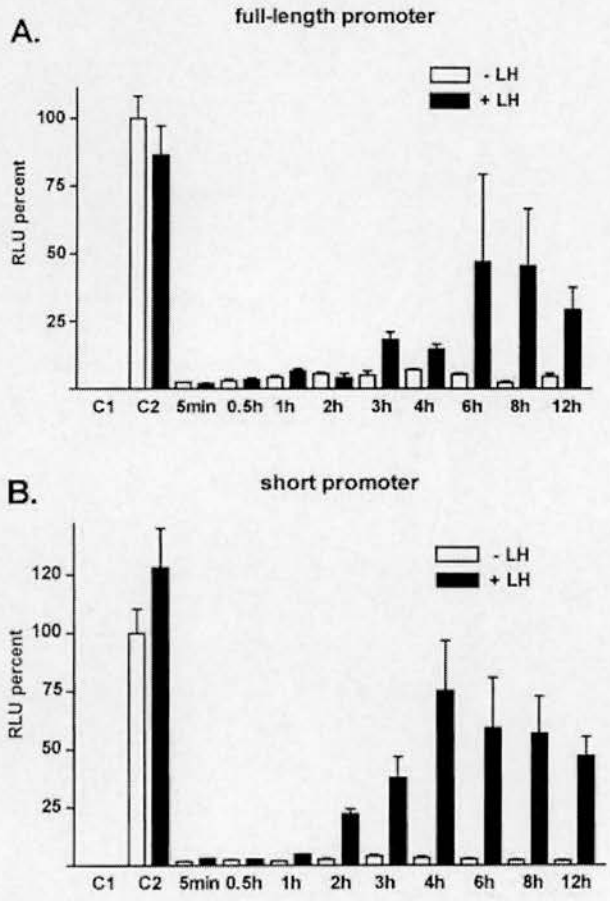

FIGURE 6. Primary cultures of bovine theca cells transfected with luciferase reporter constructs linked with either the −1245 (full-length, A) or the −315 (short, B) bovine *StAR* gene promoters. Three days after the transient transfection, cells were stimulated or not as indicated by addition of 10 ng/ml bovine LH. The amount of luciferase protein synthesized is estimated from its enzymatic activity expressed in relative light units (RLU; relative to the cotransfected CMV-β-galactosidase constitutive construct) as a percentage of the level attained with the strong constitutive promoter of the pGL3-C construct (C2) in the absence of LH. C1, luciferase activity of the empty reporter vector, pGL3-B. Data are expressed as means ± S.E.M from parallel triplicate experiments using the same batch of primary cells.

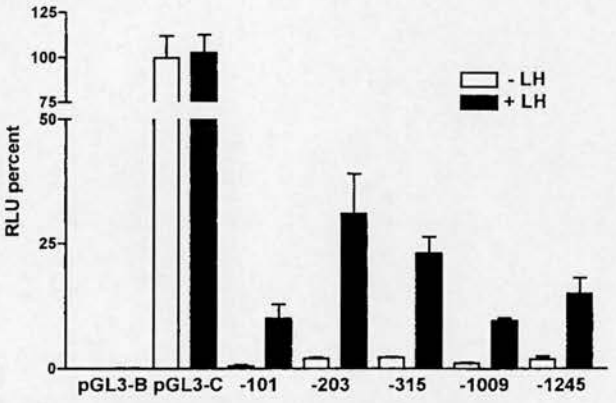

FIGURE 7. Primary cultures of bovine theca cells transfected with luciferase reporter constructs linked with different deletion fragments of the bovine *StAR* gene promoters. The promoter fragments correspond to DNA sequences extending upstream from the transcription start site to the restriction sites for BanI (−101), HpaII (−203), PstI (−315), KpnI (−1009) and BamHI (−1245), respectively. Results are expressed in relative light units (RLU) as a percentage of the luciferase activity obtained using the strong constitutive promoter construct, pGL3-C, in the absence of LH.

time-course different from that for the endogenous protein, as this will depend on the function of substrate-specific proteases. Nevertheless, it is evident that there is a plateau in luciferase expression at 4–6 h, with no further increase after this, reflecting precisely the short phase of transcriptional induction. This experiment showed that the promoter information required for this acute upregulation of transcription resides within the first 1200 bp upstream of the transcription start site, and probably within the first 315 bp of this. Use of promoter–deletion constructs confirmed what we have previously shown for this gene using a heterologous transfection system (Rust *et al.* 1998), namely that LH-induced, cAMP-dependent gene activation is maximal where the first two SF-1-responsive elements, which reside within the −315 bp immediate upstream region of the promoter, are present. It should be noted, though, that a marked LH-dependent stimulation was also evident for the very short −101 construct (Fig. 7), which does not include an SF-1 binding element, suggesting that SF-1 may not be absolutely required to mediate the stimulatory effect of LH. Other genes have also been described in the same or related cell types, for example, $P450_{SCC}$ in luteal cells (Liu & Simpson 1997) or oxytocin in granulosa cells (Wehrenberg *et al.* 1994), which also depend upon SF-1 interaction, and respond to LH stimulation. These responses, however, have a quite different time kinetic and cell specificity. Thus other factors must act in conjunction with SF-1 to encode the cellular and temporal specificity observed for the *StAR* gene. One candidate in the case of the mouse *StAR* gene is the transcription factor, CCAATT/enhanced binding protein β (C/EBPβ) (Reinhardt *et al.* 1999*a*, Silverman

et al. 1999). Support for this view is provided by a comparison of the present transfection results, using cells endogenously expressing the *StAR* gene, with the heterologous system used previously. In the previous study, the transcriptional activity induced by SF-1 attained maximally only 2% of the level reached by the same constitutively active control plasmid (pGL3-C) as that used here; this compares with greater than 50% in the present homologous system. Similarly, a constitutively active protein kinase A subunit was able to increase the SF-1-dependent transcriptional activation by only about 50% in the previous study whereas, in the homologous system, LH induced up to a 10-fold increase in reporter gene activity. The molecular mechanisms by which SF-1 could be involved in cAMP-dependent signal transduction are not yet understood. In a recent study, a potential serine acceptor site for protein kinase A (PKA) phosphorylation, and the C-terminal activation domain were both implicated in the transcriptional upregulation of the high-density lipoprotein receptor gene in rat luteal cells via SF-1 (Lopez *et al.* 1999). Also, possible mitogen-activated protein kinase phosphorylation of SF-1 has been suggested in the context of cofactor recruitment (Hammer *et al.* 1999).

The requirement for other factors to act with SF-1 in mediating cAMP-regulated signal transduction would also offer an explanation for the anomalous findings regarding specific nuclear protein-binding to the bovine StAR promoter (Rust *et al.* 1998). Of the three putative SF-1 binding motifs evident in the bovine StAR promoter (Fig. 5), only the distal element bound purified SF-1 with high affinity. The second proximal element bound SF-1 only weakly, and the most proximal element failed to show any binding activity (Rust *et al.* 1998). Yet it is the proximal promoter containing the first SF-1 motifs which, in this and other studies, appeared to mediate SF-1-dependent activation of the *StAR* gene by adenylate cyclase in (for example) humans (Sugawara *et al.* 1996), mouse (Caron *et al.* 1997*a*), rat (Sandhoff *et al.* 1998) and pig (LaVoie *et al.* 1999). Findings of a recent study using the mouse *StAR* gene promoter even suggested that an interaction of SF-1 in the proximal promoter may not be essential, and that adenylate cyclase-induced activation can be mediated by a combination of the transcription factors C/EBPβ and GATA-4 only (Silverman *et al.* 1999). This opinion is supported by findings of the present study, in which the –101 construct of the bovine *StAR* gene was able to transduce LH-stimulation to the reporter gene. This fragment does include an inverse GATA motif at position –65 (Rust *et al.* 1998).

Other regions of the promoter may be involved in the definition of expression specificity. There is evidence, from the transfection studies, for a mildly inhibitory element in the region upstream of nucleotide –315. There is also, in this region, marked nuclear protein binding unrelated to SF-1 (Rust *et al.* 1998). Furthermore, although not apparently contributing to gene activation, a very good binding site for SF-1 exists in the upstream region of the promoter at –1100 (Rust *et al.* 1998). Concerning the rapid switching off of the *StAR* gene, this might involve the activation of the negative transcription factor, DAX-1, which has been shown to interact with the mouse *StAR* gene promoter, possibly interfering directly with SF-1 binding in the proximal promoter region (Zazopoulos *et al.* 1997, Reinhardt *et al.* 1999*b*, Sandhoff & McLean 1999).

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THE EXPRESSION OF STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) IN BOVINE ADRENOCORTICAL CELLS

M.R. Nicol, H. Wang, R. Ivell*, S.D. Morley, S.W. Walker, and J.I. Mason

University Dept. of Clinical Biochemistry, RIE, Edinburgh, EH3 9YW, U.K.
*Institute for Hormone and Fertility Research, D-22529 Hamburg, Germany.

ABSTRACT

StAR protein may facilitate rapid transfer of cholesterol from the outer to the inner mitochondrial membrane, the site at which cholesterol is converted to pregnenolone by the cholesterol side chain cleavage complex. We have studied the effect of ACTH treatment on StAR mRNA and protein levels in bovine adrenocortical cells in primary culture. Cells were initially cultured for 3 days after isolation, and then treated with ACTH (10^{-8} M) for various times up to 24 hours. Northern analysis of total BAC mRNA, using a [α^{32}P]-labelled cDNA probe encoding a 5′ region of bovine StAR mRNA, revealed two principal hybridising species of 1.6 and 3.0 kb. Western immunoblot analysis revealed a principal band at 30 kDa. Levels of both StAR mRNA and protein showed an increase at 1 hour, reached a maximum at around 6 hours and declined to basal levels at 24 hours. Cortisol secretion (measured by RIA) showed a similar change over the same period. From these results it appears that StAR mRNA and protein levels in BAC are acutely regulated in concert with ACTH-stimulated cortisol secretion.

INTRODUCTION

The first enzymatic step in steroidogenesis is the conversion of cholesterol to pregnenolone ,catalysed by the cytochrome P450 side chain cleavage (P450scc) enzyme which resides on the inner mitochondrial membrane. The rate-limiting step in this process is the translocation of cholesterol from the outer to the inner mitochondrial membrane, which requires a rapidly synthesised regulatory protein. StAR protein was initially described in rat and bovine adrenal cells by using two–dimensional gel

electrophoresis to identify a family of mitochondrial proteins synthesised in response to ACTH-stimulation. StAR protein has also been found in other steroidogenic tissues (1,2) and StAR cDNA was first cloned and sequenced from mouse MA-10 Leydig tumour cells (3). MA-10 and COS-1 cells transfection with StAR cDNA, in conjunction with the P450scc system, resulted in steroidogenesis in the absence of trophic hormone stimulation (4,5). Demonstration that the inherited defect, lipoid congenital adrenal hyperplasia, is due to mutations in the StAR gene further emphasises the its role in steroidogenesis (6). StAR cDNAs have now been cloned from a variety of tissues and species including bovine luteal cells where the corresponding mRNA was found to be transcribed as 3 kb and 1.6 kb transcripts (7). In this study, we have examined the regulation of StAR mRNA and protein levels in bovine adrenocortical (BAC) cells in response to trophic hormone treatment.

METHODS

BAC cells were isolated and cultured as previously described (8). Cells were plated at a density of 5 x 10^6 per 25 cm^2 flask for mRNA studies and 1.5 x 10^6 per well of a 6-well plate for protein studies. Total RNA (25 µg) was analysed by northern blot and StAR mRNA was detected using a [α^{32}P]-labelled cDNA probe encoding a 5' region of the bovine StAR (GenBank acc.# S79908, position 210-608). Total cellular protein (25 µg) was analysed by western blot using a primary polyclonal sheep antiserum raised against a peptide fragment (amino acids 82–107) of the bovine StAR protein and a donkey anti-sheep second antibody coupled to a HRP-conjugate (The Binding Site, Birmingham, UK). The final signal was detected by chemiluminescence (SuperSignal ULTRA kit, Pierce, Rockford, USA). Cortisol contents of media were quantified using a double antibody radioimmumoassay.

RESULTS AND DISCUSSION

This study demonstrates the presence, in BAC cells, of two principal StAR mRNA transcripts of 1.6 and 3.0 kb and principal and minor protein species of 30 and 40 kDa, similar to other studies on StAR mRNA (9) and protein (10) in BAC cells. Distinctly in the present study, western blot analysis shows that freshly isolated BAC cells contain

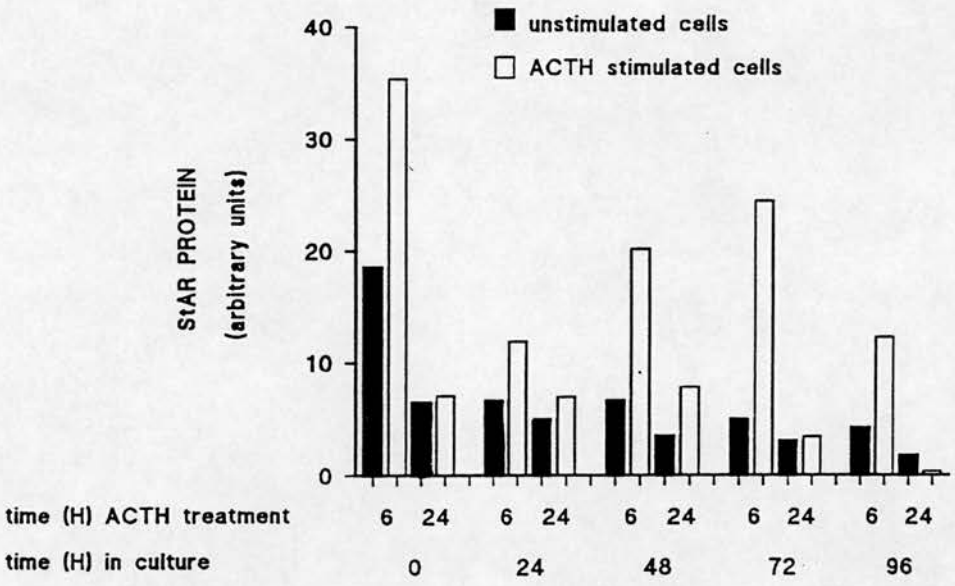

FIGURE 1

BAC cells isolated at time 0 were cultured for 24, 48, 72 or 96 hours and then treated with/without ACTH (10^{-8} M) for a further 6 and 24 hours. Quantification of Western blot analysis (30 kDa band) was made using an image analyser.

high levels of StAR protein, while these levels decrease over 3 days of culture. ACTH treatment for 6 hours following 24, 48, 72 or 96 hours of culture increases the StAR protein levels; however, when ACTH treatment is prolonged for 24 hours, the StAR protein levels return to untreated levels (Figure 1). Western blotting and northern analysis show that StAR gene expression appears to be most responsive to ACTH stimulation following 48 and 72 hours of culture (Figures 1 and 2). We therefore chose to use cells cultured for 72 hours for subsequent experiments.

Following ACTH treatment StAR mRNA levels and cortisol secretion were increased after I hour (Figures 2B and 3), whereas StAR protein levels were not increased above that in untreated cells until 6 hours of treatment (Figure 2A). This suggests that the initial steroidogenic response is not primarily dependent on the presence of high levels of newly synthesised StAR protein, although participation of a small sub-pool of new protein cannot be excluded. Instead, mechanisms, such as post-translational modification of an existing pool of StAR protein, probably initiate the initial acute steroidogenic

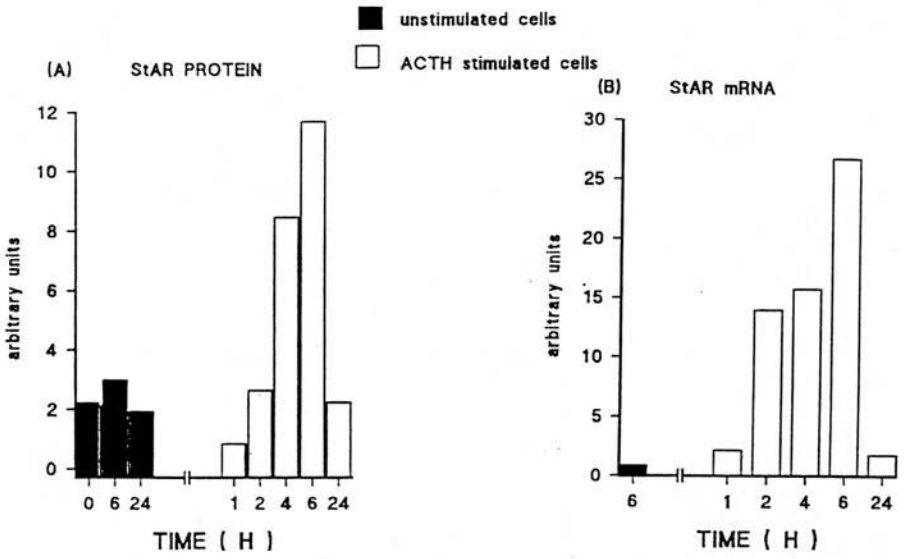

FIGURE 2

BAC cells were cultured for 72 hours after isolation, then incubated with/without ACTH (10^{-8} M) for 1, 2, 4, 6, and 24 hours. (A) Quantification of Western blot analysis (30 kDa band) and (B) Quantification of northern blot analysis (3 kb band) was made using an image analyser.

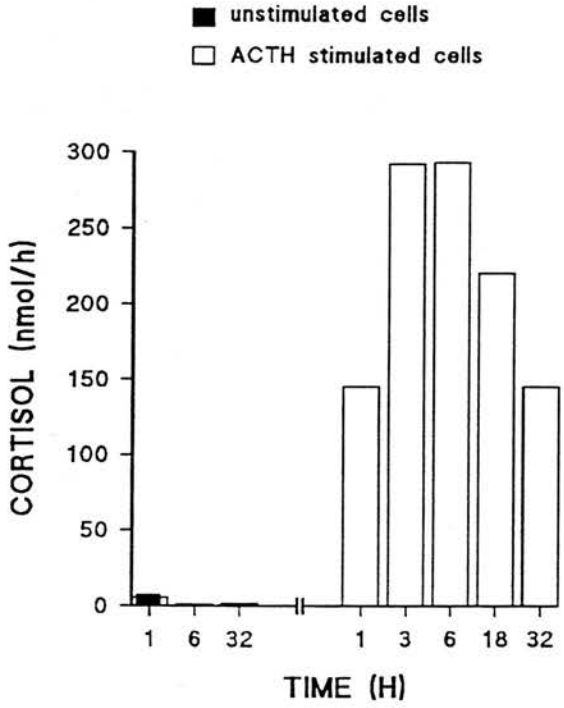

FIGURE 3

Effect of time of ACTH treatment on the rate of cortisol secretion into media overlying BAC cells (nmol/hour)

response, while newly synthesised StAR is needed to maintain steroidogenesis over a period of hours. Consistent with this hypothesis, the decreased rate of cortisol secretion observed after prolonged (24 hour) ACTH treatment is associated with decreased StAR mRNA and protein levels (Figure 3). The discordance between StAR protein and mRNA levels, and the production of cortisol at the early time–points merits further investigation.

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P95 REGULATION OF StAR PROTEIN DURING HIGH & LOW CONCENTRATION ACTH-STIMULATED STEROIDOGENESIS IN PRIMARY BOVINE ADRENAL ZONA FASCICULATA CELLS

H Wang[1], M Nicol[1], SW Walker[1], JI Mason[1], SD Morley[1], BC Williams[2].

[1]Department of Reproductive & Developmental Sciences (Clinical Biochemistry), University of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW, UK; [2]Department of Medical Sciences, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

Cyclic 3'-5' AMP (cAMP)-mediated steroidogenesis is accompanied by concomitant up-regulation of Steroid Acute Regulatory Protein (StAR). Whilst studies utilising supra-physiological levels of ACTH do indeed lead to cAMP-mediated up-regulation of StAR protein levels, the response of StAR protein to physiological concentrations of ACTH which do not stimulate cAMP (Yanazaki T. et al., 1996 Endocrinology 137:2670-75) is less clear. Therefore, the purpose of this study was to examine the correlation between cortisol secretion and StAR protein induction, in response to high (10^{-8}M) and low (10^{-12}M) ACTH concentrations in bovine adrenal zona fasciculata (ZF) primary cell cultures. Following a 0.5-24h time-course treatment of ZF cells with either 10^{-8}M or 10^{-12}M ACTH, cortisol in the medium and StAR protein in cell lysates were determined respectively by RIA or western blotting using a polyclonal sheep antiserum raised against a peptide fragment (amino acids 82-107) of the bovine StAR protein sequence (Hartung S. et al., 1995 BBRC 215:646-53). ACTH (10^{-8}M) treatment resulted in a strongly correlated induction of cortisol secretion and StAR protein levels (R=0.92), with maximal increases occurring after 4h (cortisol 144-fold; StAR protein levels 4-fold). In contrast, treatment with 10^{-12}M ACTH resulted in a maximum 24-fold increase in cortisol levels at 2h, which preceded any change in StAR protein levels, and no correlation between cortisol secretion and StAR protein induction (R<0.1). These results suggest that cortisol secretion in response to 10^{-12}M ACTH may be independent of induction of StAR protein synthesis and that steroidogenesis in response to physiological concentrations of ACTH may be mediated by intracellular signalling systems distinct from those regulating StAR protein induction.

P98 ACUTE REGULATION OF THE BOVINE GENE FOR THE STEROIDOGENIC ACUTE REGULATORY (StAR) PROTEIN IN OVARIAN THECA AND ADRENOCORTICAL CELLS.

R Ivell[1], G Tillmann[1], H Wang[2], M Nicol[2], PM Stewart[3], B Bartlick[1], N Walther[1], JI Mason[2], SD Morley[2].

[1]Institute for Hormone and Fertility Research, University of Hamburg, Grandweg 64, 22529 Hamburg, Germany; [2]Department of Reproductive and Developmental Sciences (Clinical Biochemistry), University of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh, EH3 9YW, UK; [3]Department of Medicine, University of Birmingham Queen Elizabeth Hospital, Edgbaston, Birmingham, B15 2TH, UK

Up-regulation of the steroidogenic acute regulatory protein (StAR) is implicated in the rapid synthesis and secretion of steroids by the ovary and adrenal cortex, in response to stimulation by trophic hormones of the gonadal and stress axes. In the present study we have assessed the kinetics of both StAR gene transcription and protein biosynthesis in primary cell cultures of bovine ovarian theca (OT) and adrenocortical (BAC) cells, under conditions of acute stimulation by luteinizing hormone (LH) and corticotrophin (ACTH), respectively. In both cell systems detectable up-regulation of StAR gene transcription occurs within 1-2h, reaching maxima at 4h (OT cells) or 6h (BAC cells), and returns to baseline by 12h or 24h, respectively. Specific StAR protein levels were assessed by western blotting, using a polyclonal sheep antiserum raised against a peptide fragment (aa 82-107) of the bovine StAR protein sequence (Hartung S. et al., 1995 BBRC 215:646-53), and showed a similar fast up- and down-regulation, albeit delayed by 1-2h compared to the mRNA. The response of OT cells was altogether more acute than that of BAC cells, possibly reflecting the propensity of the LH receptor to rapidly desensitise, unlike the ACTH receptor. Primary OT cell cultures were also used for fully homologous transfection studies using various deletion promoter-reporter constructs of the bovine StAR gene. Kinetic analysis of the results indicated that the acute transcriptional response resides within the proximal (-315 bp) promoter region which includes two putative responsive elements for the steroidogenic factor-1 (SF-1). More distal promoter regions may be involved in delineation of cell-type specificity by combining enhancer and inhibitory functions.